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STUDIES ON TISSUE INJURY BY HEAT

1. THE INFLUENCE OF ANOXIA

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The application of the method of perfusion of isolated tissues with saline to the study of the effect of heat in producing tissue injury, though it facilitates the detection in the perfusate of pharmacologically active substances and of enzymes, inevitably complicates the problem by providing for the cells an environment deprived of the buffering and osmotic effect of the serum proteins and with a low oxygen tension. The question at once arises as to whether the liberation of active substances into the perfusate can be attributed to the damaging effects of heat or to anoxia increased in degree by the increase of tissue metabolism brought about by rise of temperature.

We have, therefore, studied the effects of heat on the isolated lungs of guinea-pigs ventilated with oxygen, air and nitrogen. These organs even when ventilated with nitrogen, may be perfused at normal temperatures 37°–38°C. for six or more hours without showing evidence of gross injury, as indicated by the liberation of histamine into the perfusate. When ventilated with air or with oxygen the oxygen tension in the cells must be more nearly normal than in other perfused organs which cannot be supplied with oxygen in this way, but when ventilated with nitrogen they are being subjected to a degree of anoxia even more severe than are other tissues perfused with saline.

If, therefore, the degree of injury occurring at temperatures above 37° C. is not significantly increased by ventilation with nitrogen we may disregard anoxia as an important factor in causing the injurious effects produced in other tissues by perfusion at these temperatures.

METHODS.

The perfusions were carried out in a double walled metal cabinet A, Fig. 1. (34 cm. × 46 cm. × 37 cm.) with a metal door fitted with a plate glass window; the cabinet and door were insulated with 2·5 cm. fibre-board between the walls. The temperature of the cabinet was maintained at any desired level by steam from the gas heated flask B, a gas governor and bimetallic strip regulator C controlling the temperature to ± 0·1° C. Steam entered the cabinet at D; uniform distribution was ensured by the fan E and condensed water was drained at F.

Saline for perfusion from the flask G passed through drip tube H, the rate of flow being controlled at the screw clip I. The height of saline in the flask G above the level of the organ was 75 cm. which did not represent the actual perfusion pressure in cm. saline because of the slow rates of flow used (0·5 to 1 ml. per minute). The saline was heated by passing through a spiral K in water bath L which was maintained at the desired temperature to ± 0·05° C. by means of a mercury thermometer regulator M (Rawlinson, 1939) and thermionic relay and stirred by paddle N driven by the same power unit as fan E. The saline entered the cabinet at O. A Palmer oscillating pump supplied air, oxygen or nitrogen warmed to the desired temperature and saturated with water vapour by passage through a glass spiral, a sintered glass bubbler R and a second spiral P^1 which functioned as a spray-trap. The tube carrying air or other gas entered the cabinet at S and was provided within the cabinet with a small trap for condensed water. The lungs rested on a thin sheet of plastic over funnel T (4" dia.), a tube from which led through the wall of the cabinet and was provided with a hood U for the asceptic collection of perfusate. The perfusion and tracheal cannulas were held rigidly in position by thermometer clamps fixed to two short uprights on heavy bases, one of which supported the funnel.

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In all experiments a preliminary perfusion was carried out on the operation table using an accessory perfusion apparatus in which the saline was warmed by passing through a glass spiral in a bath at 38° C. The saline for perfusion contained NaCl 0·79 p.c., KCl 0·02 p.c. and $CaCl_2$ 0·02 p.c.

Guinea-pigs weighing 450–600 gm. were killed by a blow on the head. The trachea was exposed and cannulated and artificial respiration commenced with air. The front wall of the thorax was cut away, the pericardium opened, a cannula was tied into the pulmonary artery and perfusion started after opening the left auricle. The heart was excised and the trachea and lungs dissected free from the body. The upper lobes of the right or left lung were tied off and excised for the preparation of an extract. The preparation was now transferred to the cabinet after thorough washing with sterile saline.

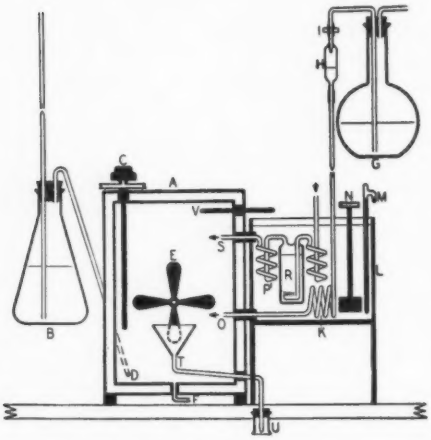

Fig. 1.

The rate of flow was adjusted and perfusate was collected continuously in half hour samples. After measuring the volume a portion was stored at 2° C. until the end of the experiment when its pH was determined by the glass electrode method. The remainder was at once boiled for histamine estimation. In these experiments, therefore, sterility of the perfusate was not so essential as in those described in the following papers. Nevertheless, precautions were adopted to avoid gross bacterial contamination. The interior of the cabinet and the tubes and spiral K in the fluid perfusion system were sterilized by steam for half an hour. The cannulas and connecting tubing, the flask G with unbuffered saline, and the accessory perfusion apparatus were sterilized by autoclaving and the instruments and operator's gloves by immersion in aqueous 2 p.c. Zephiran.

In experiments in which the lungs were ventilated with nitrogen the commercial gas was fed from a reducing valve to the inlet of the pump and to remove the residual oxygen (1–2 p.c.) passed from the outlet of the pump through a trap, two towers each containing 10 p.c. $Na_2S_2O_4$, 1 p.c. sodium anthraquinone β-sulphonate and 12 p.c. sodium hydroxide and a third containing 1 p.c. basic lead acetate which served to remove residual CO_2 and any sulphur-containing gases; the partial pressure of oxygen in the nitrogen was then less than 1 mm. When air or oxygen was used for ventilation in place of nitrogen it passed through the same system, the first two towers containing only water. The air spaces in the trap and at the head of the towers exerted a buffering action on the pump so that filling of the lung was more gradual in onset and decline than when the towers were not used. The pump was driven at 13 strokes per minute, affording time for collapse of the lung. When the preparation was put up in the cabinet its ventilation was first adjusted using the pump without the washing towers. The towers were then put in but after a few minutes at high temperatures some or all of the lobes ceased to ventilate. This condition of "shock" passed off after a variable time and ventilation then went on satisfactorily to the end of the experiment. Sometimes it was necessary to short circuit the washing towers for 2–3 minutes in order to ensure adequate ventilation which then continued satisfactorily with the towers in circuit. The perfusion was continued for 6 hours, after which the lung was cut down, drained for 20–30 minutes, squeezed free of excessive moisture with filter papers, weighed and made up into an extract for estimation of residual histamine.

Histamine Assay.

For routine tests a piece of isolated guinea-pig's jejunum was suspended in an oxygenated 7 ml. bath of Tyrode solution at 35° C. This usually gave good responses to 0·02 μg. and often to 0·01 μg. histamine. The samples of perfusate were tested in doses up to 2 ml. which could be added to the bath without any temperature effect after warming with the hand. The content of histamine was expressed as microgram of base. The content of histamine in the lung, both in the lobes excised before putting the preparation up and at the end of the experiment, was determined in boiled saline extracts as described by Feldberg and Kellaway (1937).

In order to ensure that the activity estimated in the perfusates and extracts by comparison with standard histamine solution, was in fact histamine, samples were filtered and incubated for 2 hours at 45° C. after the addition of Torantil (1 unit in 20 ml.). Since 1 unit can destroy 1 mg. histamine in 24 hours at 37° C. we used enormous excess of enzyme which in the conditions

of our experiments can destroy sufficient of the histamine present in standard solutions up to 1 in 1 million (a higher concentration than was present in any of our extracts or perfusates) to render a dose of 2 ml. without effect upon the isolated gut. In testing we usually left the dose in contact with the gut only for about 15 seconds to avoid any possible injurious effects of Torantil, but in this concentration Torantil has no immediate stimulant effect nor does it inhibit the action of a dose of 0·02 μg. of histamine added to the bath before washing out.

RESULTS.

In these experiments the degree of injury produced was estimated by the amount of histamine set free in the perfusate and drainage fluid expressed as a percentage of the total content of the perfused tissue. In order to avoid unnecessary handling of the lung with increased risk of bacterial contamination we did not weigh the organ before putting it up in the cabinet, but used the final weight of the lung after drainage and squeezing free from excess fluid, and its histamine content estimated in the final extract to calculate the original histamine content of the perfused tissue by the addition of the total amount estimated in the perfusate and drainage fluid. The total output in the perfusate and drainage fluid generally approximated within the limits of experimental error to the estimated loss calculated from the final weight of the organ and the difference in content of the initial and final extracts. The relevant data from an experiment illustrate the procedure and the observed degree of approximation between histamine lost from the organ and that estimated in the perfusate and drainage fluid.

Guinea-pig 450 gm. Isolated lung. Ventilated with air and perfused for 6 hours at 45·1° C. In 8 minutes the right upper lobes and in 15 minutes the lower left lobes were in "shock". The right lower lobe never became "shocked". Within one hour all lobes except the upper part of the right lower lobe were moving freely. For the last 4½ hours all the lobes were well ventilated. The first 9 half-hourly samples contained no detectable histamine, the tenth contained a trace, the eleventh and twelfth, 0·60 and 1·02 μg. The drainage fluid 4·5 ml. contained 0·60 μg. The total output was 2·22 μg. The initial extract made from the left upper lobe (0·50 gm.) contained 12·0 μg. per gram. The final weight of the perfused lobes was 2·49 gm. containing 11·3 μg. per gram. The percentage output of histamine was therefore $\frac{2{\cdot}22 \times 100}{(2{\cdot}49 \times 11{\cdot}3) + 2{\cdot}22} = 7{\cdot}3$ p.c. The output calculated by difference of initial and final tissue extracts was 1·74 μg. which differed from the observed output by 22 p.c.

That the activity estimated in perfusate and extracts was attributable only to histamine was demonstrated (in five experiments) by incubation of the filtered extracts and perfusates (containing at least 1:20 millions histamine) for 2 hours at 45° C. with Torantil. After such treatment the gut sensitive to 0·02 or 0·01 μg. histamine failed to react to doses of 2 ml. In the case of extracts containing from 0·3 to 0·5 μg. per ml. this result indicates that at least 97 p.c. of the activity has been specifically destroyed by histaminase; in that of perfusates containing 0·05 to 0·06 per ml. using a gut sensitive to 0·01 μg. histamine the result only indicates that at least 90 p.c. of the activity is attributable to histamine.

In one experiment at 47° C. in which perfusate containing 0·1 μg. per ml. was incubated for 2 hours with Torantil, a dose of 2 ml. failed to cause any response in a gut which gave a good contraction with 0·01 μg. histamine. In this case at least 95 p.c. of the stimulant activity must have been destroyed.

Though most of our observations were made at 45° C., at which temperature there is always an output of histamine, a few at other temperatures sufficed to show that the threshold of injury lay between 41° and 43° C.

At 41° C. the lungs ventilated either with air or nitrogen failed to give any detectable output of histamine during six hours perfusion. In one such experiment at 41·2° C. after six hours perfusion with good nitrogen ventilation, the final weight of perfused lungs was 2·49 gm. and their residual content of histamine 14·0 μg. per gm. No histamine was detected in any of the samples of perfusate.

At 43° C. there was a significant output of histamine when the lungs were ventilated either with air or nitrogen. Lungs perfused for 6 hours at this temperature and well ventilated with nitrogen weighed finally 2·53 gm. with a residual content of histamine of 8·0 μg. per gm. Histamine appeared in the perfusate at the end of the fourth hour and the total output was 1·20 μg., giving a percentage output of 5·6.

As will shortly be seen (Table 1), at 45° C. the output usually commenced in from 3½ to 4 hours and the mean percentage output from lungs ventilated with air and perfused for 6 hours was 6·51.

TABLE 1.

Percentage output of histamine from isolated lungs perfused at 45° C. for 6 hours.

Gas used for ventilation.	pH range in perfusate.	Final weight of lungs in gm.	Residual histamine content of lung in μg. per gm.	Total output of histamine. in μg.	P.c. output of histamine.	Time of commencement of output in hours.	Ventilation.
Air	6·6–5·5	1·75	12·0	1·87	8·2	3 –3½	Good from 1½ hours.
Air	7·0–6·1	2·21	10·0	1·85	7·7	3½–4	Good from 1½ hours except for right lower lobe which ventilated well from 2½ hours.
Air	6·8–6·1	2·8	13·0	2·99	7·6	4 –4½	Good from 2½ hours but right lower lobe never ventilated.
Air	6·6–5·8	2·49	11·3	2·22	7·3	4½–5	Good from 1½ hours.
Air	6·8–5·8	3·3	7·5	1·85	7·0	3½–4	Good from 1½ hours.
Air	6·9–6·1	2·05	10·0	1·25	5·7	4½–5	Good from 1½ hours but right lower lobe only ventilated from 4½ hours.
Air	7·1–6·1	2·42	18·0	2·45	5·3	3½–4	Moderate from 2 hours.
Air	6·4–5·5	1·96	15·2	1·56	5·0	3½–4	Good from 1½ hours but right lower lobe did not ventilate.
Air	6·4–5·9	2·8	12·0	1·68	4·8	4 –4½	Moderate from 1½ hours.
Nitrogen	6·3–6·0	3·8	14·0	6·75	11·3	3½–4	Good from 1 hour and right lower lobe was never in shock.
Nitrogen	6·7–6·0	2·6	14·3	4·10	9·9	2 –2½	Good from 1 hour.
Nitrogen	6·8–6·1	2·34	8·1	1·9	9·1	3½–4	Good from ¾ hour.
Nitrogen	6·4–6·0	1·73	6·0	0·77	6·9	3½–4	Good from 1½ hours.
Nitrogen	7·3–6·5	2·75	6·5	1·24	6·5	4½–5	Moderate.
Nitrogen	6·9–6·4	2·74	7·0	1·21	5·9	5 –5½	Good but right lower lobe never ventilated.
Nitrogen	6·4–5·6	2·25	5·4	0·75	5·8	4½–5	Good for 2¾ hours.
Nitrogen	6·9–6·1	2·2	10·0	1·27	5·5	4 –4½	Moderate from 1½ hours.
Nitrogen	6·9–6·1	3·2	6·5	0·78	3·6	4½–5	Good from 3 hours.

At 47° C. the output was further increased. In one experiment the lungs ventilated with air and perfused for 6 hours at this temperature weighed finally 3·65 gm. with a residual content of histamine of 5·0 μg. per gm. The output of histamine in the perfusate commenced in 2½ hours and with the drainage fluid gave a total of 4·12 μg. The percentage output was 18·4.

Table 1 shows the output of histamine in 18 experiments at 45° C. The first 9 in which the lungs were ventilated with air gave percentage outputs ranging from 8·2 to 4·8. The mean output was 6·51. In the last 9 experiments in the Table in which the lungs were ventilated with nitrogen, the percentage outputs ranged from 11·3 to 3·6, the mean was 7·17. The sum of the squares of the deviations from the respective sample means is 61·43 and with sixteen degrees of freedom the best estimate of the standard deviation in the population on the null hypothesis is therefore 1·959. The standard deviation of the difference of the two means is accordingly 0·9233 while the difference of the means is 0·66. The appropriate value of t is 0·714 corresponding to a value of P between 0·4 and 0·5.

These results do not provide evidence that there is at this temperature any significant increase in the output of histamine which can be attributed to ventilation with nitrogen instead of air. They do, however, show beyond dispute that, so far as the liberation of histamine into the perfusate is concerned, the degree of

ventilation far outweighs the nature of the gas (air or nitrogen) used for this purpose. The earlier "shock" passes off and oedema fluid (which contains no detectable histamine) is eliminated so that the lungs can expand and relax regularly the larger output which occurs as a result of perfusion with saline at this temperature.

The changes in pH in the perfusate, namely, a steadily increasing acidity, are not significantly different in the two sets of observations in Table 1 and cannot be used to differentiate them.

The importance of ventilation is also shown by a further group of 6 experiments at 45° C., the results of which are set out in Table 2. In these, we used more vigorous ventilation, short-circuiting the washing towers for periods of 5 or 10 minutes as necessary and allowing intervals of 2–3 minutes without ventilation to allow the lung to relax and force out any oedema fluid which had collected. We thus ensured strong ventilation with expansion and collapse of all the lobes in from 2½ to 3 hours giving intervals of 5 or 10 minutes of ventilation without the towers each half hour till the end of the experiment if this were necessary to maintain full ventilation. The ventilation with nitrogen in the experiments in Table 1 appeared to be favoured slightly by the small positive pressure from the cylinder on the inlet side of the pump which helped it to overcome the resistance of the washing towers. We, therefore, used compressed air from a cylinder to supply the pump when air was used for ventilation.

This more vigorous ventilation did not cause any liberation of histamine at 37° C. The lungs from a guinea-pig were strongly ventilated with air up to this standard during perfusion with saline at 37° C. for 9 hours. There was no detectable histamine in the perfusate and only 0·1 μg. in the drainage fluid at the end of the experiment. The final weight of the lung was 2·15 gm. and its content of histamine 16·0 μg per gm. The percentage output of histamine in 9 hours was therefore only 0·3 p.c.

TABLE 2.

Percentage output of histamine from isolated lungs ventilated vigorously and perfused at 45° C. for 6 hours.

Gas used for ventilation.	Final weight of lungs in gm.	Residual histamine content of lungs in μg. per gm.	Total output of histamine in μg.	p.c. output of histamine.	Time of commencement of output in hours.	Ventilation.
Air	1·39	12·0	3·08	15·6	3	Good from 3 hours.
Air	1·77	20·0	6·96	16·4	2½–3	Good from 2½ hours.
Oxygen	1·85	8·0	2·67	15·3	3 –3½	Good from 3 hours.
Oxygen	1·96	18·4	7·45	17·1	2½	Good from 2½ hours.
Nitrogen	1·45	8·0	3·15	21·4	2½	Good from 2½ hours.
Nitrogen	2·37	20·0	7·61	13·8	3	Good from 3 hours.

The results in Table 2 show that even in these conditions variability of ventilation was not wholly overcome and those experiments in which full ventilation was achieved earliest gave the highest percentage output of histamine. They indicate also that ventilation with pure oxygen neither increases nor decreases the output to any significant extent.

DISCUSSION.

Though anoxia causes grave impairment or cessation of function in the intact animal and in isolated organs, evidence is lacking for many tissues that it can give rise to structural damage or aggravate that produced by less subtly injurious agents.

The perfusion experiment is only suitable for the study of the effects of severe injury and the liberation of coagulable protein, histamine and other cell constituents signifies such injury and gives some indication of its extent.

Damage to living tissues even by moderate degrees of temperature change is severe. Leach, Peters and Rossiter (1943) observed microscopic changes only at temperatures above 50°C. when a heating iron was applied to the skin of the guinea-pig, but they review earlier work which indicates that most body cells are killed at 47°C., and even at lower temperatures if the time of heating is long enough. Injury caused by heat may, therefore, easily outweigh other injurious factors operat-

ing in perfusion experiments, but since the effects of heat can be graded from the most severe, culminating in complete disruption of the cells to the mild and reversible the possibility that the wholly abnormal conditions of perfusion may enhance minor degrees of injury cannot be neglected.

At 37° and 38° prolonged perfusion with saline causes some injury, because oedema fluid collects in the lungs; this is probably attributable to the absence of plasma proteins from the perfusion fluid. But neither the changes in permeability attending the development of oedema nor the regularly observed increasing acidity of the perfusate suffices to cause any significant liberation of histamine within six or nine hours.

In these experiments we were able to eliminate many possible sources of variation. The organs were rapidly raised to the desired temperature and the gas used for ventilation (fully saturated with water vapour at this temperature) could not cause either cooling or drying of the tissue. The steam used to keep the temperature of the cabinet constant not only provided an additional heat supply to make up for small losses, but also an atmosphere fully saturated with water vapour so that injury from drying of the surface of the organ could not occur.

There was, however, one variable which we were unable fully to control. In lung perfusions adequate ventilation is essential. The products of cell injury are liberated into the tissue spaces and only appear in the perfusate when they are forced out by ventilation. The injury caused by perfusion with saline at temperatures above 41° C. manifests itself by "shock" attributable to constriction of the smooth muscle of the bronchioles and attended by the collection of oedema fluid in the alveoli and bronchi. Only when the bronchioles relax and the collapse of the lung forces out the fluid can adequate ventilation be established.

In the main groups of experiments (Table 1) in which we ventilated the lungs with air and nitrogen respectively while they were perfused at 45° C., variation in the adequacy of ventilation was the main cause of the observed variation in the output of histamine and even when we used in the few experiments of Table 2 much more vigorous ventilation we were not able to eliminate this variable.

Our results, though not so numerous as is desirable in the presence of these large variations, are sufficient to indicate that anoxia does not belong to the group of agents which cause gross injury to tissue cells and that it does not aggravate heat injury to a significant extent.

SUMMARY.

During saline perfusion of the isolated lungs of the guinea-pig, tissue injury manifested by the liberation of histamine occurred within six hours at 43°C., but not at 41°C.

In eighteen experiments at 45°C., in nine of which the lungs were ventilated as uniformly as possible with air, and in nine with nitrogen, the latter group did not show a significantly greater output of histamine during perfusion for six hours. More vigorous ventilation with air, oxygen or nitrogen in six experiments gave larger percentage outputs of histamine during six hours perfusion at 45°, and even in these conditions the observed variations in histamine output were clearly related to variation in the degree of ventilation.

Our results show that anoxia does not cause gross injury to tissue cells and suggest that it is unlikely to aggravate heat injury to a significant extent.

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STUDIES ON TISSUE INJURY BY HEAT

2. THE LIBERATION OF ENZYMES FROM THE PERFUSED LIVER

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The object of these studies was to ascertain the effects of cellular injury by moderate degrees of rise of temperature. Since we were especially interested in the liberation of enzymes it seemed desirable, before experimenting with complex structures such as limbs, to study a relatively homogeneous tissue known to be rich in enzymes.

We have, therefore, perfused the isolated liver of the cat with saline at temperatures ranging from 38°–50°C. In the preceding paper (Kellaway and Rawlinson, 1944), we have shown that the liberation of histamine from the isolated lungs of the guinea-pig is not significantly increased by anoxia more extreme than that which attends the perfusion of an isolated organ with saline. In evaluating the results of our present work we shall discuss the extent to which injury attributable to heat is superimposed upon that caused by depriving the cells of the normal buffering and osmotic effects of the plasma proteins.

METHODS.

For these experiments we used the isolated liver of the cat perfused through the portal vein with saline containing NaCl 0·79 p.c., KCl 0·02 p.c., $CaCl_2$ 0·02 p.c. and in some experiments $NaHCO_3$, M/133.

The animals were anaesthetized with sterile 1 p.c. chloralose (10–11 ml. per kg.) which was injected into an internal saphenous vein exposed under local anaesthesia. The preparation was made as described for the dog by Feldberg and Kellaway (1937), only a portion of the liver (left lateral, right lateral and caudate lobes or all three) being used. A boiled saline extract was made from pieces of the remainder of the liver for histamine estimation.

After preliminary perfusion at 38° C. on the operation table the preparation was set up on a small glass stand in the funnel T of the perfusion cabinet described and figured in the preceding paper. The level of saline in the flask G was 60 cm. above the perfused organ.

As soon as it was put up in the cabinet, the liver was quickly brought to the required temperature by perfusion for 20 to 30 minutes at 20 to 30 ml. per minute before commencing the collection of perfusate at a rate of 1 to 2 ml. per minute.

In perfusion experiments of four or more hours duration it was essential to exclude bacterial contamination and procure sterile perfusate. The interior of the cabinet and the tubes and spiral K in the fluid perfusion system were sterilized by steam for half an hour. The cannulas and connecting tubing, the flask G with unbuffered saline, the accessory perfusion apparatus, towels and ligatures were sterilized by autoclaving. The bicarbonate was added to the saline after sterilization.

The preparations were made aseptically; the animal's hair was clipped closely and the skin prepared with an aqueous solution of Zephiran (2 p.c.). The instruments were sterilized by prolonged immersion in 2 p.c. Zephiran. It was necessary for the operator to wear gloves. The perfusate was sterile when these precautions were adopted.

Histamine assay. Samples of perfusate and liver extracts were assayed for histamine on the isolated guinea-pig jejunum as described in the preceding paper (Kellaway and Rawlinson, 1944). Incubation for 2 hours at 45° C. with Torantil (1 unit to 20 ml.) was used to discover whether the activity of the perfusates and extracts estimated by comparison with histamine in μg. of base was attributable to histamine itself.

Histaminase. Samples of perfusate were mixed with an equal volume of 1: 5 millions histamine solution and incubated for 2 hours at 45° C. They were then boiled and their histamine content estimated on the isolated gut preparation.

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Hydrogen-ion concentration. A portion of each of the samples of perfusate was stored at 2° C. until the end of the experiment when its pH was estimated by a glass electrode and two standard buffers.

Protein and glycogen. A 2·00 ml. sample of perfusate was diluted in a centrifuge tube with 6·00 ml. absolute alcohol and 4·00 ml. trichloracetic acid (20 p.c.). After standing for 2 hours at 2° C. the mixture was centrifuged. The precipitate of protein and glycogen was re-suspended twice in absolute alcohol, re-centrifuged and finally dried and weighed to give the total protein and glycogen. The protein content was determined by weighing the precipitate obtained when a 2·00 ml. sample was diluted with 2·00 ml. trichloracetic acid (20 p.c.). Glycogen was determined by difference and was qualitatively confirmed by the iodine reaction.

Inorganic phosphate. The supernatant fluids from the above were used for inorganic phosphate determinations by a modification of the method of Atkins and Wilson (1926). Special attention was paid to the precautions mentioned by Berenblum and Chain (1938). The standard phosphate solutions contained the same concentrations of trichloracetic acid as the samples: 0·50 to 1·00 ml. of supernatant fluid was diluted to 50·0 ml. with distilled water; 1 ml. molybdate reagent and 0·20 ml. acidified 1·2 p.c. stannous chloride were added. The estimations were made using a Dubosecq colorimeter when the blue colour had reached a maximum (7–10 min.). Some of our results for inorganic phosphate were obtained from the controls used in the determination of alkaline phosphatase.

Alkaline phosphatase. The alkaline phosphatase activity of the samples was estimated both with and without magnesium ions as activator and the stock substrate recommended by Shinowara, Jones and Reinhart (1942): 1·00 ml. sample, 2·00 ml. stock substrate, 1 drop of 25 p.c. $Mg\,Cl_2.6\,H_2O$ and 0·50 ml. toluene were placed in 10 ml. tubes closed with rubber stoppers. This series of tubes, together with another series without magnesium were incubated at 36° C. for 16 hours. Trichloracetic acid 20 p.c. (3·00 ml.) was added to each tube. The mixtures were well shaken and filtered after standing for five minutes. Inorganic phosphate was determined on an unincubated series treated in a similar manner, phosphatase activity being measured by difference.

Catalase. The catalatic activity of perfusate was determined by iodometric titration (Bancroft and Elliott, 1934). Amounts of samples varying between 0·10 ml. and 3·00 ml. depending on their activities were diluted to 200 ml. before addition of H_2O_2 (7 mg.). It was found essential to titrate aliquots at a fixed time interval (5 min.) after the addition of potassium iodide. Test portions (10 ml.) were taken at 0, 2, 4 and 6 minutes, potassium iodide solution was added at 1½, 3, 4½ and 7 minutes and titration with 0·005 N sodium thiosulphate solution was commenced at 6½, 8, 9½ and 12 minutes respectively.

Esterase. Esterase activity was estimated by the method of Green (1934) slightly modified: 20 ml. M/50 phosphate buffer of pH 8·0, 0·25 ml. ethyl acetate, 2 drops of 0·2 p.c. phenolphthalein and 2 ml. of sample were mixed thoroughly in a 100 ml. erlenmeyer flask and stoppered. Incubation was carried out at 37° C. for 2 hours, the flasks were then cooled rapidly in the refrigerator and immediately titrated with 0·01 N sodium hydroxide solution.

The method depends upon the measurement of increase in acidity which is not specific; a boiled sample could not be used as a control because of the bicarbonate present nor could the substrate be omitted since allowance for non-enzymatic hydrolysis had to be made. In our control we therefore replaced the sample before incubation by 2 ml. of distilled water but added 2 ml. of incubated sample immediately before titration. These control values were small in comparison with those given by liberated esterase.

Proteolytic activity. This was estimated using gelatine substrate and cysteine hydrochloride as activator: 6·00 ml. sample, 6·00 ml. gelatine substrate (2·5 p.c. gelatine, 0·05 p.c. cysteine hydrochloride, M/15 phosphate buffer pH 7·0) and 1 ml. toluene were mixed thoroughly, stoppered and incubated at 36° C. for 40 hours. 3·00 ml. portions were then pipetted into 60 ml. absolute alcohol, 7 drops 0·2 p.c. thymolphthalein added and the solution titrated with N/10 aqueous sodium hydroxide.

RESULTS.

The aseptic perfusion of the cat's liver with saline at temperatures from 38° to 50° C. causes swelling of the organ and reticulation of its surface (attributable to loss of pigment). The perfusate when it has become free from blood is sooner or later coloured by escaping pigment, turbid from cell detritus and sometimes from glycogen and contains increasing amounts of protein coagulable by heat, histamine and other muscle stimulating substances, phosphate and enzymes. In some cases globules of fat are observed. These changes occur in 2 to 4 hours at 48° C., in 3 to 5 hours at 45°–43° C. whereas at temperatures between 40°–38° C. from 6 to 10 hours are required to produce them.

Hydrogen-ion Concentration.

Our results are derived from twenty-three experiments at the following temperature levels: 50° C. (1), 48° C. (8), 45° C. (3), 41·6°–42·8° C. (5), 40° C. (2), 39° C. (2) and 38° C. (2). In fifteen, sodium bicarbonate was present in the perfusion fluid.

The observed changes in pH are illustrated by the curves of Fig. 1, those labelled *b* being from experiments in which bicarbonate saline was used. In these there is an initial rise, then a steep fall in pH usually followed by a more gradual rise. The fall occurs earlier at high than at low temperatures. Except in one of eight experiments, that figured at 42·7° C., these changes are significantly absent when there is no bicarbonate in the perfusion fluid, the net result being a gradual increase in the acidity of the perfusate throughout the experiment.

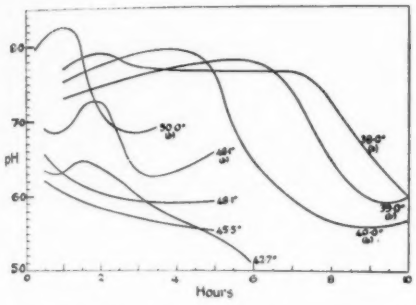

Fig. 1. Changes in H+ concentration in perfusate from the cat liver. Ordinates, pH: abscissae, time in hours. b denotes bicarbonate-saline.

Pigment.

We have noted the time of output of pigment in most of these experiments. At 50° C. traces were present in the first quarter hour, the output reached a maximum in 2½ hours and thereafter decreased. At 48° C. traces were present in the perfusate at the end of the first hour, the output reached a maximum during the third or fourth hour and had greatly diminished by the fifth hour. At 45° C. the output commenced at about 2 hours and reached a maximum in from 5 to 5½ hours. At 43° C. output began at 3½ hours and no observable maximum was recorded in 5½ to 6 hours. At 41·6° C. the output commenced at 4½ to 5 hours. At 40° C. it first appeared in 5 hours and reached a maximum in 7 hours. At 39° C. the output commenced at about 7 hours and reached maxima at 9 and 11 hours. At 38° C. it first appeared at 9 hours but maxima could not be determined with certainty in experiments lasting 10 and 12 hours.

In two experiments at 39° C. quantitative colorimetric comparison was made of the samples (freed from glycogen and filtered) with that in which the output was maximal. In one experiment the percentages from the eighth to the twelfth hour were 22, 36, 56, 100 and 72 and in the other from the sixth to the twelfth hour were 4, 13, 38, 100, 68, 90 and 54.

Protein and Glycogen.

We noted the output of coagulable protein in the boiled samples from twenty-five experiments and accurately determined the time at which a maximum was reached in twelve experiments in which phosphatase determinations were made. At 50° C. pigment and protein appeared simultaneously and reached maxima in 2½ hours. At 48° C. coagulable protein appeared during the first hour and reached a maximum in the third or fourth hour. At 45° C. it appeared in about 2 hours and reached a maximum in 5 to 6 hours. At 42° C. it commenced at 2½ to 3 hours and reached a maximum at the end of the sixth hour. At 40° C. it appeared in the fifth hour and reached a maximum in the seventh hour. At 39° C. it appeared in the seventh hour and reached a maximum in the ninth to the eleventh hour.

Gross turbidity attributable to the liberation of glycogen only occurred in a few experiments. At 48° C. it was observed in the third and fourth hours, at 45° C. in the fourth, fifth and sixth hours and at 41·6° C. early in the seventh hour.

These general observations indicate that coagulable protein is set free early, closely followed by pigment and after several hours by glycogen.

The output of protein and glycogen was studied quantitatively in five experiments at 48·1° C. in samples between 2½ and 4½ hours. The maximum output of protein observed was 1·01 p.c. and of glycogen was 0·53 p.c. The condensed protocol of a single experiment illustrates the kind of data obtained.

Weight of liver, 45·9 gm.; perfused 5 hours at 48·1° C. The half hour samples at 3 and 3½ hours were investigated. At 3 hours the total protein and glycogen was 0·395 p.c., the protein 0·355 p.c. and glycogen 0·040 p.c. When the sample was digested for 2 hours at 37° C. it showed a loss in total precipitate of 0·015 p.c. At 3½ hours the total protein and glycogen was 0·555 p.c., protein 0·315 p.c. and glycogen 0·240 p.c. The loss in total precipitate by digestion was 0·265 p.c. most of which must be attributable to loss of glycogen.

The wide variation in the content of glycogen in liver cells (up to 20 p.c.) explains why it was observed in significant amounts in only a few experiments. In these it was probably present in the liver in high concentration. Failure to detect it is also in part explained by the fact that it is broken down by the phosphorylase and amylase systems set free in the cell and that this enzymatic degradation continues in the perfusate even at 2° C.

Histamine and other Muscle Stimulant Substances.

Our results are based upon thirty-six experiments at the following temperature levels: 48° C. (11); 45° C. (8); 43° C. (2); 42° C. (5); 41° C. (3); 40° C. (3); 39° C. (1); 38° C. (3).

The cat's liver contains but little histamine. In our observations the initial content ranged from 0·35 to 4·0 μg. per gm. tissue. In 5 experiments we showed that these estimates were too high because only 82 to 86 p.c. of the muscle stimulant activity of our boiled extracts and perfusates could be attributed to histamine. This is illustrated in the following protocol.

Cat 4·4 kg. Isolated liver perfused with saline containing M/133 $NaHCO_3$, for 5½ hours at 48° C. The last 5 half hourly samples were filtered and pooled after testing. The pooled material contained muscle stimulant substance (estimated on the guinea-pig gut) equivalent to 0·225 μg. histamine per ml. After incubation with Torantil (1 unit per 20 ml.) for 2¾ hours at 45° C. its activity was equivalent to 0·04 μg. histamine per ml. The addition of more enzyme and further prolonged incubation failed to reduce this value. The filtered 1: 20 liver extract possessed stimulant activity equivalent to 0·05 μg. histamine per ml. and after incubation for 2¾ hours at 45° C. with Torantil (1 unit per 20 ml.) its activity was reduced to a little less than 0·02 μg. histamine per 2 ml. In both cases only about 4/5 of the muscle stimulant activity could be attributed to histamine.

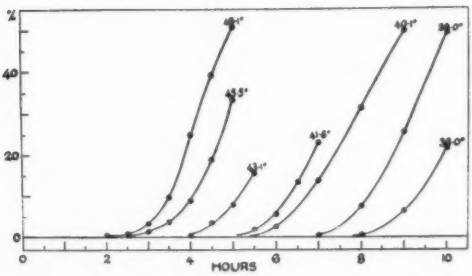

Fig. 2. Output of histamine and other plain muscle stimulating substances in perfusate from the cat liver as percentages of the total content of perfused organ. Ordinates, percentage: abscissae, time in hours.

Our experiments on the liberation of stimulant substances showed some variation both in the time at which it commenced at any one temperature and in the percentage output up to a given time. In 11 experiments at 48° C. stimulant substances were first detected in measurable amounts either in the second or early in the third hour. The percentage outputs to the end of the fourth hour were 6·0, 9·1, 14·3, 20·0, 21·2, 24·1, 25·0, 30·3, 30·6, 47·9 and 82.

In five experiments at 45·4° C. the output commenced in the third or early in the fourth hour and reached by the end of the fourth hour 3·3, 4·4, 8·9, 26·2 and 26·7 p.c. In two experiments at 43·1° C., two at 42·8° C. and one at 42·7° C. output began in the fourth or early in the fifth hour and at the end of the fifth hour 7·9, 25·4, 5·2, 5·7 and 17·6 p.c. were reached.

In three experiments at 41·6° C. output first occurred late in the fifth or sixth hour and reached at the end of the sixth hour 0·4, 5·6 and 6·7 p.c. In three experiments at 40° C. measurable activity was first observed in the sixth hour and by the end of the sixth hour had reached 2·5, 6·6 and 8·3 p.c.

Despite these large individual variations, the percentage output curves of Fig. 2 may be taken as representing the whole group of experiments at temperatures ranging from 38° C. to 48° C. They show a time-temperature relationship in the degree of injury; at higher temperatures the output commences after a shorter lag, and rises more steeply than at lower temperatures.

The liberation into the perfusate of muscle stimulant substances commences later than that of coagulable protein, pigment, phosphate or any of the enzymes which we have studied. In the experiments at 48° C. we were able to determine the time of maximum output which occurred regularly at about the fourth hour. In experiments at 40° C. perfused for 9 and 11 hours it occurred at 8 and 9 hours and in a single experiment at 39° C. the maximum was at 10 hours. At the intervening temperatures although the time of perfusion was sufficiently long to obtain maxima for most of the other substances which we have investigated we were not able to observe maxima for stimulant substances.

Inorganic Phosphate.

The output of phosphate was studied in seventeen experiments; at 48° C. (7), 45° C. (2), 42·5° C. (1), 42·0° C. (1), 40° C. (2), 39° C. (2) and 38° C. (2). It is expressed as micrograms of phosphorus liberated per minute.

Even at low temperatures during the early hours of perfusion there is a significant output of inorganic phosphate (9 to 56 μg. P per minute). This steady loss, which is not prevented by the addition of phosphate to the perfusion fluid, is greatly increased at 48° C. The following condensed protocols of perfusions for five hours at 48·1° C., the first two without added phosphate and the last two with saline containing M/600 phosphate illustrated this point. The initial figure

is the final weight of the perfused organ and it is followed by the values for successive half hourly samples. In the last two experiments the added P has been deducted.

43·4 gm.; 43, 25, 24, 21, 25, 47, 89, 100, 76, 59.
45·9 gm.; 8, 5, 9, 22, 26, 65, 50, 44, 36, 38.
46·2 gm.; 60, 30, 63, 93, 90, 131, 124, 112, 62, 38.
42·2 gm.; 16, 8, 3, 13, 4, 15, 56, 136, 127, 62.

The high initial values in the first samples may be attributed to the slowing down after the period of rapid perfusion to bring the organ up to the desired temperature.

The output of inorganic phosphate (μg. P. per min.) in six typical experiments has been plotted against the time in Fig. 3. They show superimposed on an initial leak from the perfused organ a time-temperature relationship in the effects of injury. At high temperatures the lag is shorter and the curves rise more steeply to their maxima than at lower temperatures.

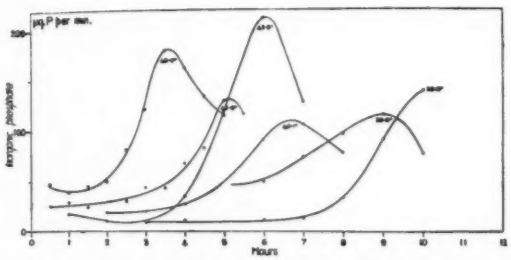

Fig. 3. Output of inorganic phosphates. Ordinates, micrograms P per minute: abscissae, time in hours.

Alkaline Phosphatase.

Our results were obtained from twelve experiments: 48° C. (2), 45° C. (2), 42·5° C. (1), 42° C. (1), 40° C. (2), 39° C. (2) and 38° C. (2). Alkaline phosphatase activity in the samples has been calculated as milli-micrograms of phosphorus liberated per minute at 36° C. by hydrolysis of sodium β-glycerophosphate. Thus, if x μg. phosphorus is liberated per ml. sample in t_1 minutes incubation at 36° C. and the rate of flow of perfusate is v/t ml. per minute, then $\frac{xv}{t_1 t}$ 103 milli-micrograms will be liberated per minute.

A duplicate series of observations has been made with magnesium ions as activator; this causes considerable increase in the activity of all samples, particularly the earlier ones. In some experiments the maxima were reached more rapidly when magnesium was added, giving the true maxima for the output of enzyme. The later occurring maxima in experiments without added magnesium may indicate the later liberation of co-enzymes.

Fig. 4 shows the output in five selected experiments. In only two of these (40·1° C. and 45° C.) are the maxima appreciably earlier in the presence of magnesium. In most of the experi-

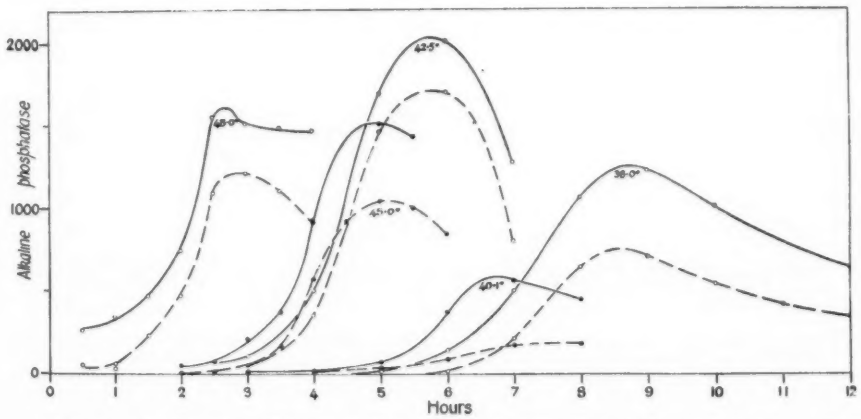

Fig. 4. Alkaline phosphatase activity of perfusate from the cat liver. Ordinates, milli-micrograms of phosphorus (see text): abscissae, time in hours. Continuous lines represent samples activated with magnesium; broken lines represent unactivated samples.

ments the addition of magnesium reveals the presence of phosphatase even in the first hour of perfusion and the degree of activation is much greater than at later periods when the perfusate shows very great activity and when co-enzymes are probably being liberated.

Catalase.

Our results are based on twenty-six experiments at the following temperature levels: 50° C. (1), 48° C. (3), 45° C. (7), 43° C. (5), 41·6° C. (2), 40° C. (3), 39° C. (2), 38° C. (3). The catalatic activity of the samples of perfusate has been expressed as values of K derived from the first order equation, $K = \frac{2{\cdot}3}{t} \cdot \log \frac{a}{a-x} \cdot \frac{200}{S}$, where a and (a–x) represent the H_2O_2 concentrations at time 0 and t minutes respectively and $\frac{200}{S}$ represents the dilution factor, S being the volume of sample used for the estimation. The values obtained do not take into account either the weight of perfused tissue or the rate of flow (approximately 1 ml. per minute but varying in individual experiments from 10 to 20 p.c.).

During the first hours of perfusion before heat injury was manifest small amounts of catalase could be detected in the perfusate at all temperatures (1–3 units), the accuracy of the method permitting the estimation of even these small amounts to 0·1 unit.

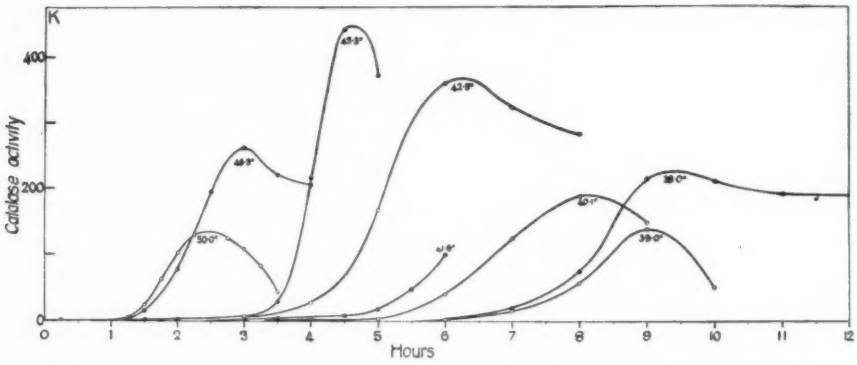

Fig. 5. Catalase activity of perfusate from the cat liver. Ordinates, K values (see text)· abscissae, time in hours.

The output curves shown in Fig. 5 are the records of individual experiments selected as representing the group at each temperature. The output commences progressively earlier as the temperature rises between 38° C. and 50° C. and reaches its maximum more rapidly as the temperature increases. At the lower temperatures the output tends to be more prolonged. The weights of the organ in experiments figured were 50° C. (49 gm.), 48·3° C. (23 gm.), 45·3° C. (58·8 gm.), 42·5° C. (53·7 gm.), 41·6° C. (47 gm.), 40·1° C. (42·3 gm.), 39° C. (32·8 gm.), 38° C. (68·1 gm.).

Esterase.

We performed 23 experiments at the following temperature levels: 50° C. (1), 48° C. (8), 45° C. (3), 41°–43° C. (5), 40° C. (2), 39° C. (2), 38° C. (2).

The esterase activity is expressed as micro-equivalents (μE) of ester capable of being hydrolysed per minute at 37° C. by the perfusate; e.g. if b ml. N normal sodium hydroxide were required to titrate the acid liberated when c ml. perfusate was incubated at 37° C. for m minutes and if v ml. perfusate flowed in t minutes then μE per minute is $\frac{Nbv}{cmt} \cdot 10^3$. This expression of results is possible because liver esterase hydrolysis is of zero order mechanism and thus independent of substrate concentration. In expressing our results we have therefore taken into account the rate of flow of perfusion though not the weight of organ perfused.

Fig. 6 shows the output of esterase in micro-equivalents of ester against the time of perfusion in hours. Each curve is the record of a single experiment selected as representing its group. The higher the temperature of perfusion between 38° C. and 50° C., the earlier the commencement of output and the attainment of a maximum. At and below 40° C. the rise to and fall from a maximum is slow.

During the first half hour of perfusion at temperatures below 45·5° C. no detectable esterase was present in the perfusate and even at 48° C. the output during the first hour only reached 0·07 in one experiment. The protocol of the following experiment, which is not graphed, may be regarded as an effective control for observations at higher temperatures.

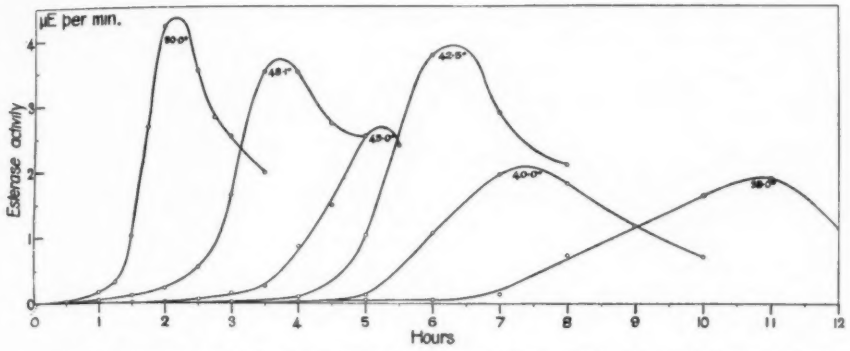

Fig. 6. Esterase activity of perfusate from the cat liver. Ordinates, μE units (see text): abscissae, time in hours.

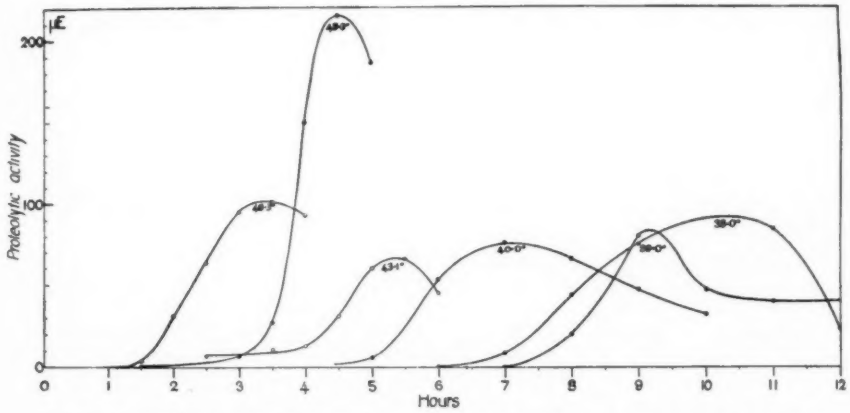

Fig. 7. Proteolytic activity of perfusate from the cat liver. Ordinates, μE units (see text): abscissae, time in hours.

Weight of perfused organ 49·5 gm., temperature 38° C., perfused 10 hours. Hourly samples gave following values: 0·00, 0·00, 0·02, 0·03, 0·04, 0·03, 0·03, 0·18, 0·98 and 2·11.

Our observations of the change in hydrogen-ion concentration during perfusion led us to consider the possibility that autolytic processes favoured by low pH might be responsible for part of the observed liberation of esterase. In four experiments at 48° C. in which bicarbonate was present the maxima (obtained graphically) were reached in 3·40, 3·50, 3·72 and 4·30 hours (mean 3·73, standard deviation 0·35) and in a similar group without bicarbonate the maxima were reached in 3·23, 3·29, 4·14 and 4·25 hours (mean 3·73, standard deviation 0·47). These results though few in number afford a strong indication that no significant acceleration or retardation in the output of esterase can be attributed to the presence of bicarbonate.

Proteolytic Enzymes.

Our results are based upon twenty-five experiments at the following temperature levels: 48° C. (3), 45° C. (7), 43° C. (3), 42° C. (2), 41·6° C. (2), 40° C. (3), 39° C. (2) and 38° C. (3).

The activity of the samples which represents the combined activity of proteinases and peptidases has been expressed as micro-equivalents of base-titratable groups liberated per ml. perfusate when digested with protein substrate for 40 hours at 36° C. The accuracy of the estimations which is much less than for the other enzymes studied is ± 3 μE units. This expression of results takes no account either of the weight of organ perfused or the volumes of samples.

Fig. 7 shows the output in six selected experiments. At higher temperatures the output commences earlier and reaches a maximum earlier than at lower temperatures where the level of high output is more prolonged.

A small but significant output occurred during the first hour in 10 of the twenty-five experiments and seven of these were at temperatures above 45° C. At temperatures of 40° C. or lower there was generally no detectable output for four or five hours but in one experiment at 40° C. and one at 39° C. there was an output of 5 to 14 μE during the first five hours.

DISCUSSION.

The liver perfused through the portal circulation is most suitable for these studies. Its parenchymal cells are all of one type, they have a rich and varied content of enzymes and they come directly into contact with the perfusion fluid so that their constituents can be liberated directly into it.

In these experiments it was essential that the desired temperature be attained rapidly; otherwise considerations of a relationship between the intensity of heating and the time required for the liberation of cell constituents would be invalid. If we make the reasonable assumption that the efficient circulation of saline throughout the tissue eliminates conduction as a limiting factor, then thermal equilibrium will be established between perfusion fluid and cells and the equation $t = \frac{L}{r} \ln \frac{\theta_1 - \theta_0}{\theta_1 - \theta}$ will give the time t in minutes for the organ (weight L and specific heat of unity) at initial temperature θ_0 to reach the temperature θ when perfused at the desired temperature θ_1 by saline at the rate of r ml. per min. Perfusion for twenty to thirty minutes at twenty to thirty ml. per minute is according to this equation adequate to bring the organ (of average weight 50 gm.) to within 0·1° C. of the desired temperature, neglecting any heat gained from the air of the cabinet. Maintenance of temperature at the slow rate of perfusion was achieved mainly by the surrounding air which, saturated with water vapour, possessed a high heating capacity and prevented the drying of the surface tissue.

The first sample at the slow rate after the initial rapid rate of flow invariably showed a slightly higher output of inorganic phosphate than the second sample; this suggests that grossly different rates of flow may influence the time-temperature relationship. This behaviour may be characteristic only of readily diffusible substances, for none of the other constituents studied showed the same effect. Even if it were true that the absolute rates of output were different at consistently higher or lower rates of flow, conclusions from a study of the relative rates of output of a number of constituents would still remain valid. The slight variations in rates of flow in our experiments did not cause any detectable difference in the rates of output at any temperature.

The hydrogen-ion concentration and buffering capacity of the perfusion saline and their possible effects on the degree of injury must next be considered, particularly since changes of temperature are known to affect the acid-base balance. Without the addition of substances of high molecular weight and the capacity of binding acids and bases it is not possible to buffer saline so that it will maintain a steady pH throughout perfusion. Most inorganic salts in concentrations sufficient for effective buffering would upset the osmotic balance of the system; phosphates can only be used in low concentrations owing to the presence of calcium in the perfusion fluid and sodium bicarbonate requires carbonic or similar acid to attain a pH value around 7·4. We do not know to what extent the hydrogen-ion concentration within the cell can be influenced by that of the perfusion fluid. The

results of the two sets of esterase experiments in one of which bicarbonate was used to increase the alkali reserve of the perfusate showed that there was no gross difference in the time of liberation of esterase at 48°C., which makes it appear that acid production within the cells either does not contribute to tissue injury or that extra-cellular alkali has no significant influence on the hydrogen-ion concentration within the cells.

The pH curves of Fig. 1 demonstrate the general effects which we observed. An initial rise which corresponded to the fall in output of inorganic phosphate occurred in all the bicarbonate-saline experiments. Decrease in the output of acidic substances may not be the sole explanation of this, but it may have been determined in part by change in the bicarbonate-carbonate system. This is unlikely to have been caused by heat, because the passage of saline through the bath and cabinet even at 50°C. without passing through the organ did not cause any appreciable change in pH; possibly the system was influenced by carbonic anhydrase. Another possibility is that basic substances were liberated early, but only one of the eight experiments without bicarbonate supports this view. After this initial pH rise we observed a rather sudden fall which ran parallel to the liberation of the cell constituents studied, but in experiments without bicarbonate in which the pH values were lower initially such rapid changes did not occur during the early hours of perfusion, possibly because the liberated phosphate acted as buffer.

The perfusions at lower temperatures attained somewhat lower pH minima. At temperatures above 45° it would be expected that the more rapid output of cellular products would lower the pH values. This was not the case, probably because acid-producing enzyme systems were inhibited above this temperature. Bradley (1938) states that the acids produced in control digests of liver in order of amounts are phosphoric, carbonic, fatty acids and lactic. In these experiments we observed that the phosphate output during the perfusion closely followed the changes in hydrogen-ion concentration. Towards the end of perfusion in fatty livers large amounts of fatty acids were observed.

The liberation of cell constituents during saline perfusions at normal temperatures (37°–38°C.), although not occurring for about eight hours, must be regarded as indicating cell damage, probably of autolytic nature, and it becomes important to decide whether this process is a determining or a subsidiary factor at higher temperatures. The curves shown in Figs. 2 to 7, which present detailed evidence of the liberation of some cell constituents give, by inspection, only a general idea of the time-temperature relationship.

Except in the case of histamine, we do not know the initial content in the liver and, as emphasized by Bodansky (1937), care must be exercised in selecting a suitable measure for the rates of the process. Osterhout (1918) showed that if the form of the function did not alter with temperature, then the reciprocal of the time to reach a specified output was proportional to the rate for first or higher order processes. With the constituents whose total amounts in the organ could not be estimated, we know either the output in unit time or the enzyme activity of perfusate samples at various times, and for comparison it becomes necessary to select a readily defined point common to all curves. Time values which might be selected are (i) where the output shows the first sudden increase, (ii) the mid-point of the first inflection before the curve becomes linear, (iii) where the output curve first becomes linear, and (iv) where the output curve reaches a maximum. Each activity value plotted in Figs. 2 to 7 represents the average during the time from the preceding sample and not the value at the actual time. The slopes of the resultant curves are thereby somewhat modified and the time values (i) to (iv) will show corresponding small variations. The time of maximum output, however, should show least variation from this cause and can be ascertained with greatest accuracy; we have, therefore, selected it for use in Figs. 8b to 8f. Though the

points plotted in Fig. 8 were derived from the individual experiments in Figs. 2 to 7, the curves have been drawn after taking into account the whole of our results.

Comparison of the curves in Fig. 8 reveals several facts. At 38° none of the substances investigated reached maximum output until at least nine hours. The rapid increase in output of alkaline phosphatase, esterase, proteolytic enzymes and, to a lesser degree, of histamine, as the temperature approaches 40° to 41° C. indicates that this is a critical level for heat injury. At 40° C. the maxima were

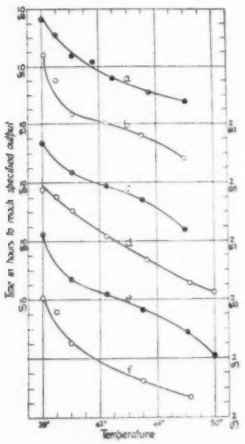

Fig. 8. Composite graph of time to specified output against temperature (a) 10 p.c. output of histamine. Maximum output of (b) inorganic phosphate, (c) alkaline phosphatase, (d) catalase, (e) esterase, (f) proteolytic enzymes. Values of ordinates, in hours, for each curve are designated by the corresponding letter. Abscissae, temperature in degrees Centigrade.

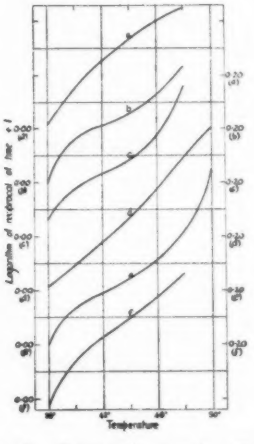

Fig. 9. Composite graph. Ordinates, 1 + logarithm of reciprocal of time specified in Fig. 8 (values designated by letter for each curve). Abscissae, temperature in degrees Centigrade which is proportional to reciprocal of Absolute temperature over this range.

reached in the following order: phosphatase, phosphate, proteolytic enzymes, esterase and catalase. The extreme differences between these times was more than one hour, and is, therefore, significant; above 43° C. these differences were approximately thirty minutes, so that with time plotted directly in hours characteristic trends for the individual curves are not obvious.

We have, therefore, sought to ascertain whether there is an exponential relation between rate of liberation and temperature by plotting (Fig. 9) from the curves of Fig. 8 the logarithms of the reciprocal of the specified times against the temperatures in degrees Centigrade. These functions have been suggested by consideration of the Arrhenius equation, $\frac{d \ln k}{dT} = \frac{-\mu}{RT^2}$, where k is the rate constant of the process, T the absolute temperature, R the gas constant and μ an empirical temperature characteristic. Integration of the equation, substitution of the reciprocal of the specified time t as proportional to k and conversion to common logarithms gives $\log \frac{a}{t} = \frac{-\mu}{2.303\,R}\left(\frac{1}{T} + C\right)$, where a and C are constants.

This equation is satisfied if a straight line is obtained by plotting $\log \frac{1}{t}$ against $\frac{1}{T}$. For catalase and proteolytic enzymes where we have calculated μ values we have used the equation in the form, $\mu = \frac{2 \cdot 303 \, RT_1 \, T_2 \, (\log t_1 - \log t_2)}{T_2 - T_1}$ where t_1 and t_2 are the times to reach the specified outputs at the absolute temperature T_1 and T_2.

Over the range of experimental temperatures the reciprocal of the absolute temperature is proportional to the temperature in degrees Centigrade and to facilitate comparison we have used the latter scale in Fig. 9.

The curve for histamine (Fig. 9a) though it is more acute towards 38° C. does not provide definite evidence for the assessment of the degree of damage at normal temperature. Histamine appears later in the perfusate than most of the other cell constituents studied, and in view of its low molecular weight and ready diffusibility in the free state, it seems likely that the energy necessary to dissociate it from combination within the cell must be the limiting factor in its liberation. If this be so, we should expect a straight line, but it must not be forgotten that part of the muscle stimulant activity estimated as histamine is attributable to the presence of some other substance or substances.

The low but definite output of inorganic phosphate even from the commencement of perfusions at 38° C. indicates that it has little difficulty in leaving the cell and, therefore, should serve as a sensitive indicator of minor degrees of cell damage. Simple diffusion, however, with energy of activation of about 3 kcal, is not likely to become a limiting factor in liberation because the energy of translation from within the cell must be greater under all conditions. Fig. 9b shows a rapid increase in the rate of output as the temperature reaches 40° C., and we may, therefore, infer that slight rises of temperature above normal contribute to cell damage to a significant extent. The inorganic phosphate which is liberated into the perfusate is not all initially present in this form within the cell; it is produced to a large extent by enzymatic cleavage and the number of processes involved makes it difficult to offer any interpretation of the shape of the curve at higher temperatures because, although some enzymes concerned are inactivated or destroyed, non-enzymatic hydrolysis is increased.

The curve for alkaline phosphatase (Fig. 9c) supports our conclusion that as the temperature rises above normal the increase in the rate of liberation is greater than can be attributed to cell damage by saline perfusion. This enzyme appears to exist in the cell in the form in which it appears in the perfusate, and requires little energy for its liberation. The acute curvature above 44° to 45° C. suggests that at higher temperatures the time to attain maximum output is no longer a true measure of the rate of liberation, since the apparent maximum output must occur earlier when destruction or inactivation of the enzyme is in progress. The denaturation of proteins and the inactivation of enzymes have been found to possess large heats of activation (over 40 kcal) and their reaction velocities may, therefore, be expected to be so low as to exert little influence in our experiments even at the higher temperatures, but on account of corresponding increases of entropy of activation the reactions are still able to proceed at appreciable rates (Eyring and Stearn, 1939).

Bodansky (1939) demonstrated that above 42° to 43° C. bone phosphatase activity was rapidly decreased; this close agreement with our observations is striking when the difference in conditions and source of enzyme are remembered.

Dounce (1943) has shown that for rat liver, alkaline phosphatase is normally distributed between the cell nuclei and protoplasm so that the nuclei (approximately 6 p.c. of the total cell volume) possess 192 p.c. of the total activity. A similar distribution probably holds for cat liver and it must be expected that dif-

ferent amounts of energy would be required to liberate the enzyme from these two sources. If the rate of liberation from the nuclei were significantly slower than that from the cytoplasm then it would modify the output but if rapid liberation occurred from the nuclei then it would have little or no influence on the rate of liberation into the perfusate.

Catalase (Fig. 9d) shows two straight lines intersecting at about 44·3° C. with μ values of about 10 kcal and 27 kcal and Q_{10} values of 3·2 and 3·8 respectively. At first sight this might appear to indicate that the liberation of catalase is caused by an injury process attributable to saline perfusion whose rate increases exponentially with rising temperatures. In the light of the evidence provided by other cell constituents studied that heat contributes a large part to the observed injury, an alternative explanation of the behaviour of catalase is probable; that either the energy of its dissociation from cellular combination, its conversion into a diffusible form or the energy of translation across the membrane is the rate-determining factor in the range 38° to 50° C. That the output is governed by single factors supports our assumption that the reciprocal of the time to the specified output is a true measure of the rate constant. The point of intersection at about 44° C. probably indicates that another of the factors mentioned above, comes into operation above this critical temperature. Dounce (1943) was able to detect only a trace of catalatic activity in the cell nuclei of rat liver, and its presence in the cytoplasm and not in the nuclei of the cat's liver would agree with the conclusions that its liberation is determined by single factors operating within the cell and independent of the initial damage caused by saline perfusion. The maintenance of a straight line at temperatures up to 50° C. shows that catalase is not destroyed by heat in the conditions of our experiments.

The curve for esterase (Fig. 9e) is closely similar to that of alkaline phosphatase and the same general interpretation can be applied to it. Destruction must be occurring above 45° to 46° C., and the rate of output may also be affected if the distribution in the cat liver of enzyme between nuclei and protoplasm is similar to that in the rat, 50 p.c. of total activity being in the nuclei (Dounce, 1943). The sharp inflection above 38° supports the conclusion that severe injury attributable to the effects of heat is superimposed upon the initial damage caused by perfusion with saline.

The curve for proteolytic enzymes (Fig. 9f) has a less acute inflection above 38° C.; it is nearly linear between 42° and 46° C., with μ about 18 kcal and $Q_{10} = 3·1$. This suggests that our activity measurements are those of a single enzyme, proteinase, and shows that there is no definite heat destruction up to 48° C.

These considerations of an exponential relationship between the rate of liberation of cell constituents and temperature has enabled us to make several pertinent conclusions of a comparative nature and also demonstrates, as other workers have done for many biological systems, that in spite of apparent complexity there exists under the specified conditions of test only a limited number of processes capable of acting as limiting factors.

SUMMARY.

After about seven hours perfusion of the isolated liver of the cat with saline at 38° C., histamine, inorganic phosphate, alkaline phosphatase, catalase, esterase and proteolytic enzymes begin to appear in the perfusate; this is indicative of cell damage.

The rapid increase in the rate of liberation of inorganic phosphate, alkaline phosphatase, esterase, proteolytic enzymes and to a lesser degree of histamine when the temperature approaches 40° to 42° C. is considered to be greater than can be accounted for by the normal exponential increase of the process operating at 38° C.

The liberation of catalase and histamine just above 38° C. is slower than that of the other constituents studied; the liberation process for catalase satisfies the Arrhenius equation and it is, therefore, concluded that the rate-determining steps are characteristic of the enzyme.

The gross deviation from the Arrhenius equation by alkaline phosphatase above 44° to 45° C. and by esterase above 45° to 46° C. demonstrate that irreversible heat inactivation is commencing. Catalase shows no such destruction up to 50° C.

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STUDIES ON TISSUE INJURY BY HEAT

3. ISOLATED LIMB PREPARATIONS

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Whether a toxic agent plays any part in producing systemic effects in severe burns and scalds, apart from those attributable to bacterial infection, is still an open question, but if such an agent is responsible it is likely as Peters has pointed out (Leach, Peters and Rossiter, 1943), to originate not from totally destroyed tissue cells but from those at the periphery of the burn which have suffered only partial damage.

The perfusion experiment enables tissues to be brought rapidly to a given temperature level and to be maintained there within $\pm$ 0·1° C. without any temperature gradient. It also provides fluids (perfusate and oedema) which are not so complex as blood and so simplifies the search for toxic cell constituents or products of enzyme action within damaged cells or in the tissue spaces, and is convenient also for the estimation of enzymes liberated in heat injury.

Two objections may be offered to its use: that anoxia may be a major influence in the production of injury during perfusion at temperatures above normal and that the abnormal conditions of perfusion in respect to osmotic and hydrogen-ion balance may be more important in causing injury than the increased temperature. These objections have been considered in the two preceding papers.

The experiments reported here are a preliminary study of the effects of moderate degrees of rise of temperature in the perfused limbs of the guinea-pig and cat.

METHODS.

Our experiments were carried out by saline perfusion of hind limb preparations from guinea-pigs and fore limb preparations from cats. The physiological saline contained NaCl 0·79 p.c., KCl 0·02 p.c. and $CaCl_2$ 0·02 p.c. and for the fore limb in addition M/150 $NaHCO_3$. The perfusions were carried out in the cabinet described in an earlier paper (Kellaway and Rawlinson, 1944). The preparations were suspended over the funnel for the collection of perfusate by loose ties proximal to the paws. The perfusion cannula was held rigidly in position by a thermometer clamp fixed to a short upright on a heavy base. A similar stand supported the cross bar from which the limbs were suspended and the funnel. The level of the saline was 125 cm. above that of the perfused organ.

Though the cabinet, the perfusion apparatus and the fluid itself were sterile and the operator wore gloves, in making the preparation we considered full asepsis less important than the possibility of contributing to heat injury of the skin by chemical agents used to sterilize it. We, therefore, after our early experiments, abandoned the use of Zephiran (2 p.c.) for the skin preparation.

Guinea-pig hind limb. The animal was killed by a blow on the head, the abdomen was opened, the inferior mesenteric artery was ligated and the rectum tied off in the pelvis. A cannula directed distally was tied into the descending aorta below the level of the renal arteries and perfusion was commenced. The body wall and the spinal column were now cut across freeing the preparation which consisted of the hind limbs, the pelvis, the bladder with the uterus and adnexa in the female and the vesiculae seminales and testes in the male. In some experiments the uterus was tied off and excised together with the upper portion of the vagina. In preparations from males the testes which in any case were not perfused were excised. After about a litre of fluid had been perfused through it the preparation was set up in the cabinet and perfused at 37° C. for half an hour at the fast rate (10–15 ml.

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per minute). The temperatures of the water bath and cabinet were now raised to the required degree and, after 20 minutes perfusion at the fast rate to raise the temperature of the preparation to the desired level, perfusion was continued at 1 to 2 ml. per minute and half hourly samples were collected for from four to six hours.

Cat fore limb. The animals weighing 2·5–4 kg. were generally anaesthetized by the intravenous injection of sterile 1 p.c. chloralose solution (10–11 ml. per kg.) injected under local anaesthesia into an internal saphenous vein. In a few experiments to exclude the possible influence of this anaesthetic we used dial liquid intraperitoneally (0·2 ml. per kilogram) or open ether. An incision was made extending from the level of the manubrium sterni parallel to the midline to the level of the tenth rib and then sweeping outwards to near the midline posteriorly. This was continued vertically upwards to the level of the shoulder and across the neck to join the commencement of the first incision. Sterile towels were used to clip off the body edges of this incision. The muscles attached to the posterior border of the scapula were now divided without ligating any bleeding points. The pectorals were divided; the arteria thoracica anterior was ligated and the axillary artery tied close to the thoracic wall. A cannula was inserted distally and perfusion started, the axillary vein being at once opened. Interscapulo-thoracic amputation was now completed and the long flaps of skin sutured loosely so as to cover the scapula and the exposed muscular tissues. The preparation was now hung up in the cabinet and perfused at 10–15 ml. per minute, about a litre of perfusion fluid being used, before the perfusion proper at 1 to 2 ml. per minute was commenced. In this way the limb was brought quickly to the desired temperature.

Histamine assay. Samples of perfusate were assayed for histamine on the isolated guinea-pig jejunum. To ascertain whether the acidity of the perfusate estimated in micrograms of base by comparison with standard histamine solutions was attributable wholly to histamine we incubated them for 2 hours at 45° C. with Torantil (1 unit in 20 ml.) and again tested them on the isolated guinea-pig jejunum.

Histaminase. Samples of perfusate were mixed with an equal volume of 1: 5 millions histamine solution and incubated for 2 hours at 45° C. They were then boiled and their histamine content estimated on the isolated gut preparation.

Cardiodepressant activity. We tested for this using adenosine as standard on the heart of the atropinized guinea-pig by the method of Drury, Lutwak-Mann and Solandt (1938). In some experiments the perfusion fluid contained sodium cyanide M/100 or M/40 neutralized with HCl Before testing, hydrocyanic acid was removed from the perfusate by addition of HCl and boiling (Kellaway and Trethewie, 1940). In other experiments the perfusate was collected at 95° C. or for the first fifteen or twenty minutes of each half hour sample at 95° C., and for the remaining fifteen or ten minutes at room temperature. The guinea-pig preparations were from males.

Active perfusate was incubated with filtered saline extract of the perfused cat liver for 2 hours at 40° C. to ascertain whether its activity could be attributed to adenyl compounds or other substances which the enzyme systems in this extract could inactivate. The extract in a dose of 0·4 ml. added to 5 ml. of 1 in 10,000 adenosine completely inactivated it in 1½ hours so that 1·1 ml. (100 μg. adenosine q.p.) was without effect on the guinea-pig heart.

Alkaline phosphatase and inorganic phosphate. The alkaline phosphatase activity of the perfusate from fore limb preparations and incidentally the inorganic phosphate were estimated by the method described in the preceding paper (Rawlinson and Kellaway, 1944) but since the perfusates were much less active than those from liver, 2·00 ml. sample and 1·00 ml. stock substrate were used instead of the volumes indicated there.

Lipase. The lipolytic activity of perfusate from the fore limb of the cat was estimated as follows: 10 ml. phosphate buffer (0·02 M and pH, 8·0); 2·00 ml. triacetin (3·5 p.c.); 2·00 ml. serum as activator; 2 drops phenolphthalein and 2·00 ml. sample. The mixture was incubated at 37° C. for 2½ hours and a control similar to that described for esterase was used. The increase in acidity was determined by titration with 0·01 N sodium hydroxide.

Proteolytic activity. The proteolytic activity of perfusate from fore limb preparations was estimated as described in the preceding paper. In some experiments freshly prepared cat-muscle globulin (1·5 p.c.) adjusted to pH 7·0 with a phosphate buffer was used as substrate. The protein was not purified by repeated solution and precipitation as it was desirable to maintain unaltered its normal highly labile character. The action of proteolytic enzymes present in it was accounted for in control experiments.

RESULTS.

(1) *Guinea-pig Hind Limbs.*

In this preparation we demonstrated the liberation of histamine and of histaminase at temperatures between 45° C. and 50° C. Though actively cardiodepressant adenyl compounds could not be detected in the perfusate at temperatures between 43° C. and 53° C., cardiodepressant activity which was not destroyed by incubation with liver extract was found in experiments at 43° C. and 45° C.

Histamine.

Table 1 shows the output of histamine in μg. from three groups of preparations: from males, from females and from females in which the uterus had been excised.

TABLE 1.

Liberation of histamine from the perfused hind limbs of the guinea-pig.

Sex.	Temperature.	Time at which histamine first appeared.	Range of output in μg. per min.	Total output in 4 hours in μg.
Males.				
	50° C.	2½–3 hr.	0·013–0·014	0·8
	48° C.	2½–3 hr.	0·011–0·025	1·39
	48° C.	2 –2½ hr.	0·017–0·057	4·60
	48° C.	2 –2½ hr.	trace –0·03	1·46
	47° C.	2 –2½ hr.	0·011–0·013	1·45
Females.				
	50° C.	1½–2 hr.	0·013–0·056	5·27
	48·4° C.	1½–2 hr.	trace –0·028	2·47
	48° C.	1 –1½ hr.	trace –0·043	3·90
	48° C.	1½–2 hr.	0·04 –0·077	7·93
	47° C.	3 –3½ hr.	trace –0·017	0·51
	45° C.	3 –3½ hr.	trace –0·008	0·73
	37° C.	—	—	0
Females with uterus excised.				
	48° C.	1 –1½ hr.	trace –0·025	1·92
	48° C.	2½–3 hr.	0·011–0·015	1·21
	48° C.	1½–2 hr.	0·016–0·056	5·33

For a duration of perfusion of 4 hours (after an initial perfusion of one hour at 37° C.) it appears that injury occurs even at 45° C., and that at 48° C. there is a regular ouput of histamine commencing at the end of the second hour. The preparations from female guinea-pigs appear to exhibit an earlier and larger output than those from males. They have in addition to the skin a second source of histamine in the uterus and when this organ is excised the preparations should approximate in behaviour to the male type. Our observations though suggestive are too few to be decisive.

Histaminase.

A similar difference was suggested by the behaviour of preparations from male and female guinea-pigs in regard to the output of histaminase. It was not liberated during perfusion at 37° C. but in preparations from female guinea-pigs containing the uterus it was generally present by the end of the first hour of perfusion at 45°–48° C. The following condensed protocols of four experiments show the percentage loss of histamine from successive half hourly samples of perfusate mixed with equal parts of 1 in 5 million histamine and incubated for two hours at 45° C.

48° C.:	41	33	0	0	0	0	0
48° C.:	35	23	17	0	0	0	0
47° C.:	55	38	38	27	20	11	0
45° C.:	0	17	23	23	0	0	0

These results can only be taken as affording qualitative evidence of the presence of histaminase in the perfusate because some samples of perfusate after boiling or incubation at 47° C. for 2 hours before the addition of histamine inhibited the stimulant action of from 10–25 p.c. of the added histamine. Unboiled perfusate failed to mask any of the added histamine The following condensed protocol shows the apparent percentage loss of added histamine for seven half hourly samples of perfusate obtained during perfusion of a preparation from a female guinea-pig at 47° C. for 3½ hours and illustrates these anomalies.

Untreated perfusate	0	0	0	0	0	0	0
Boiled perfusate	17	0	0	0	0	7	0
Incubated perfusate	10	10	25	17	25	8	0
Perfusate incubated with histamine	42	58	83	67	50	33	33

In these experiments no trace of histaminase was detected during the first hour of perfusion at 37° C. In two preparations from male guinea-pigs of similar age and weight and in two from female guinea-pigs after the uterus had been excised no histaminase could be detected in any of the samples during four hours perfusion at 48° C. The following experiment shows that the uterus is a source of histaminase.

The uterus of a female guinea-pig was perfused through the aorta for 25 minutes, excised, minced and ground thoroughly for 30 minutes with finely powdered silica. A saline extract was made and divided into two parts, to one of which was added an equal volume of sterile saline (A) and to another an equal volume of 1 in 5 million histamine (B). These two mixtures were halved, one part of each being boiled at once (A and B) and the other (A^1 and B^1) after incubation for two hours at 45° C. Histamine assay on the isolated jejunum gave the following values in μg. per ml.: A 0·09, A^1 0·04, B 0·18 and B^1 0·04 μg. The extract without added histamine lost 56 p.c. whereas that with added histamine lost 78 p.c. of its activity. In making the extract part of the liberation of histamine from the ground-up tissue probably only occurred during boiling (Trethewie, 1938) and if histamine in the tissue were not susceptible to the action of histaminase it would explain the smaller loss in the extract to which no histamine was added.

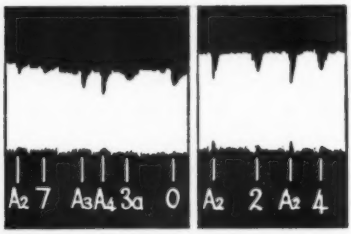

Fig. 1. Records from right auricles from two atropinized guinea-pigs.

Left hand panel: tests of perfusate and oedema fluid from guinea-pig hind limbs at 43° C.

At 7 1 ml. of 3½ hour sample (boiled), at 3a 1 ml. of 1½ hour sample (unboiled) and at 0 1 ml. oedema fluid (boiled).

Right hand panel: tests of perfusate from guinea-pig hind limbs perfused at 45° C.

At 2 1 ml. of 1 hour sample boiled, at 4 1 ml. of 2 hour sample boiled.

At A_2, A_3 and A_4, 2, 3 and 4 μg. adenosine.

Cardiodepressant Substances.

We performed 9 experiments at 53° C., 50° C. (2), 48° C., 47° C., 45° C. (2), 43° C. and 37·5° C. In those at 53° C. and 50° C. sodium cyanide M/100 or M/40 was present in the perfusion fluid; in the remainder, part or all of the perfusate was collected at 95° C.

In none of these experiments were any active adenyl compounds detected in the perfusate but some of the samples in experiments at 45° C. and 43° C. had a cardiodepressant effect upon the atropinized guinea-pig heart. This resembled the effect later to be described for perfusate from the cat fore limb. The cardiodepressant action was more prolonged than that of adenosine (Fig. 1) resembling rather the effect produced by sodium citrate. Incubation of the samples with cat liver extract at 40° C. for 2 hours did not diminish their cardiodepressant activity. Activity was found in equal degree in perfusate collected at 95° C. and at room temperature and was unaffected by boiling.

The sensitivity of the heart preparation to this cardiodepressant activity is paralleled by its sensitivity to adenosine but the activity can only be approximately matched with adenosine. The condensed protocols of three experiments show the activity of successive half hour samples in micrograms adenosine per ml.

45° C.: tr, 1, 1, 1·5, 1·5, 1·5, 1, 1, 0, 0, 0, 0.
45° C.: tr, tr, 2·5, 2, 0, 0.
43° C.: 0, 0, tr, tr, 1, 1·5, 1·5.

Though the perfusate collected during perfusion at 37·5° C., 47° C. and 48° C. had no demonstrable activity, the subcutaneous oedema fluid obtained, when the preparation was cut down, was active. At 47° C. 1 ml. was equivalent to 4 μg. adenosine, at 45° C. to 2 μg. at 43° C. to 4 μg. and at 37·5° C. after perfusion for six hours the oedema in a dose of 2 ml. was equivalent to 1 μg. adenosine. This activity like that of the perfusate was unaffected by boiling or by incubation with cat liver extract.

(2) *Cat Fore Limb.*

In this preparation we studied changes in hydrogen-ion concentration during perfusion and showed that histamine was liberated into the perfusate. Though cardiodepressant adenyl compounds could not be detected at temperatures between 37·5° C. and 50° C., cardiodepressant activity which was not decreased by incubation with cat liver extract was observed in the perfusate at temperatures between 42° C. and 50° C.

We also studied the liberation of inorganic phosphate, of alkaline phosphatase, of lipase and of proteolytic enzymes.

Hydrogen-ion Concentration.

Our results are derived from sixteen experiments, in two of which bicarbonate was omitted from the saline, and eleven experiments in which the cabinet was at a higher temperature than the perfusion fluid.

The two experiments at 45·5° C. in which bicarbonate was not used showed a steady fall in pH during the first three and a half hours of perfusion and thereafter but little change up to the end of six hours; the range in these was from 6·95 to 5·84 in one and from 6·60 to 5·70 in the other. In two corresponding experiments with bicarbonate in the perfusion fluid the pH ranges were 8·00 to 7·40 and 8·00 to 6·87. In the first of these the pH remained steady for one and a half hours and then underwent a gradual fall to the end of the fifth hour, in the other the pH fell steadily from the beginning.

In general at temperatures from 47° C. to 50° C. there was relatively little change in pH during the perfusion. In one experiment at 47·1° C. the pH of successive half hour samples were 7·79, 7·75, 7·70, 7·64, 7·66, 7·64, 7·52, 7·47, 7·48, 7·47, 7·42 and 7·36. At 44° C. to 45° C. the change in pH was much greater; for example, in one experiment at 45° C. the pH of successive samples were 7·71, 7·85, 7·64, 7·34, 7·01, 6·79, 6·60, 6·46, 6·45, 6·41, 6·38 and 6·34. At lower temperatures again the change in pH was less; in an experiment at 37·5° C. the pH range was from 7·77 to 7·56.

Histamine.

Our results are based on eighteen experiments at temperatures between 37·5° C. and 50° C. and on thirteen in which the cabinet was raised to temperatures between 42° C. and 52° C. but the perfusion fluid was only heated to 20° C. We thus attempted to produce severer injury to the surface than to the deeper tissues. Doubtless during the early part of the experiments this object was attained for with the cabinet at 50° C. or 52° C. the temperature in the perfusion cannula was 40° C. to 41° C. However, after six hours perfusion the temperature measured deep within the preparation was only 1·5° C. to 2° C. less than that of the cabinet.

At temperatures between 43° C. and 50° C. histamine was liberated in considerable amounts but though all the muscle-stimulant activity which we estimated on the isolated gut could be destroyed by histaminase, its stimulant action on the gut in some samples of perfusate was significantly reduced by the presence in the perfusate of a heat-stable muscle-relaxing substance. This was revealed when we tested every sample of perfusate for histaminase as we did in a number of experiments. We then found that in some samples in which there was no detectable histamine an immediate reduction in the titre of added histamine took place though no further loss occurred during incubation for two hours or more at 45° C. The smooth muscle-relaxing substance could only be detected early in the perfusion before histamine was liberated in significant amounts. In late samples, in some of which histamine was present in concentrations ranging from 1: 2 to to 1: 7 millions, titration by the vaso-depressant effect on the blood pressure of the atropinized cat gave results in good accord with those on the isolated intestine of the guinea-pig. Except for these later samples our estimations of histamine are possibly somewhat too low. Doubtless also the times at which histamine first appears in the perfusate in measurable amounts are a little earlier than our observations indicate.

There was much variation in the actual amounts of histamine liberated at any one temperature and our experiments fall into two classes: those in which histamine appeared in the perfusate early and gave during six hours a considerable output and those in which at the same temperatures it appeared later and reached in the late samples only a fifth to a tenth as high a level. In this last group the presence of an inhibiting muscle-relaxing substance was more obvious and interfered somewhat with the titration.

The experiments with a high output are illustrated by the following five condensed protocols in which are given the amounts of histamine in micrograms in successive half-hourly samples during perfusion for six hours:

50° C.: 0·6, 4·4, 11·8, 9·0, 16·1, 17·2, 20·5, 24, 24·2, 22, 24, 23·1.
48° C.: 0, 0, 0, 0·5, 0·8, 1·1, 2·7, 4·0, 3·9, 4·4, 5·2, 5·5.
47·1° C.: 0, 0, 0, 0·5, 0·3, 0·4, 0·4, 1·1, 1·7, 2·9, 2·8, 3·1.
45° C.: 0, 0, 0, 0·6, 0·9, 0·9, 2·5, 2·0, 2·0, 3·5, 5·0, 6·3.
42·4° C.: 0, 0, 0, 0, 0, 0·4, 1·0, 1·7, 1·3, 2·3, 2·0, 4·6.

The output at 50° C. commenced at once, at 48° C. to 45° C. at the end of the second hour and at 42·4° C. at the end of the third hour. The output in the first experiment though large is not unusually so, for in seven experiments with the cabinet at 52° C. or 50° C. and cool perfusion fluid the maximal values observed for a half hourly sample were 40·5, 25·0, 12·3, 12·1, 10·3, 3·5 and 1·8 μg.

The type of experiment in which the histamine output was small is illustrated by the following five condensed protocols:

50° C.: 0, 0, 0, 0, 0, 0·5, 0·8, 2·1.
47·9° C.: 0, 0, 0, 0, 0, 0, 0, tr, 0, 0·8, 0·4.
46·9° C.: 0, 0, 0, 0, 0, 0, tr, tr, 0·2, 0·3, 0·7, 0·6.
45·5° C.: 0, 0, 0, 0, 0, 0, 0, 0, 0·4, 0·5, 0·7, 0·9.
43·8° C.: 0, 0, 0, 0, 0, tr, tr, tr, tr, 0·3, 0·3, 0·5.

At 41° C. the output of histamine did not commence until the sixth hour, in one experiment the total output in the sixth, seventh and eighth hours were 0·7, 0·9 and 0·9 μg. At 40° C. histamine first appeared in the seventh hour the output in one experiment in the seventh and eighth hours being 0·8 and 1·6 μg. At 38° C. in one experiment histamine first appeared with an output of 3·8 μg. in the eighth hour.

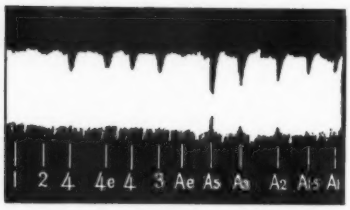

Fig. 2. Record from right auricle of an atropinized guinea-pig. Tests of perfusate from the fore limb of a cat at 47·8° C.

At 1, 2, 3 and 4 0·5 ml. of half hour, one hour, one and half hour and two hour samples of perfusate (boiled).

At 4e 0·55 ml. of a mixture of 0·5 ml. 2 hour sample + 0·05 ml. liver extract incubated 2 hours at 40° C.

At A_1, $A_{1·5}$, A_2, A_3 and A_5, 1, 1·5, 2, 3 and 5 μg. adenosine.

At Ae 1·1 ml. mixture of 1 ml. 1 in 10,000 adenosine with 0·1 ml. liver extract incubated for 2 hours at 40° C.

Cardiodepressant Substances.

The results reported here are based upon 14 experiments at temperatures between 37·5° C. and 50° C. Despite thorough preliminary perfusion at 37° C. to remove as much as possible of the inactivating enzyme in the plasma, the use of rapid rates of flow to shorten the period of action of enzymes set free from the muscle and collection at 95° C. to limit the time of action of the inactivating enzyme, we have not been able to demonstrate even traces of active adenyl compounds in the perfusate. However, at temperatures between 42° C. and 50° C. some of the samples regularly exhibited cardiodepressant activity which was more prolonged than that of adenosine upon the atropinized guinea-pig heart preparation (Fig. 2). The degree of this activity was as great in samples collected at room temperature as in those collected at 95° C. even when the former were allowed to stand two hours before boiling and filtering for test. Even boiling in strongly acid solution caused no loss of activity nor did incubation for two hours at 40° C. with saline extracts of cat liver or skeletal muscle rich in enzymes capable of inactivating large amounts of adenosine.

The following condensed protocols show the cardiodepressant activity as equivalents of adenosine in μg. per ml. in half hourly samples.

50° C.:	0, 5, 3, 2, tr, 0, 0, 0.
50° C.:	0, 4, 3, 4, 5, 2, 0, 0.
48° C.:	0, 4, 6, 6, 4·5, 5, 4·5, 5, 3, 2·5.
47·8° C.:	0, tr, 5, 5, 4, 4, 3, 3, 2·5, 1, tr, 0.
45·3° C.:	0, 0, 0, 1·5, 4, 4, 5, 5, 6, 5, 2, 1·5, 1, 0.
45° C.:	0, 0, 0, 0, 0, 2, 4, 4, 6, 6, 4, 4.
42° C.:	0, 0, 0, 0, 0, 0, tr, tr, 2, 2, 3, 3·5, 2, 2.
38° C.:	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0.
37·5° C.:	0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0, 0.

Similar cardiodepressant activity was also present in fluid collected by scarifying the oedematous subcutaneous connective tissue which after perfusion for two to four hours had swollen to a thickness of 1 to 1·5 cm. This fluid had either the same as or greater activity than the most active samples of perfusate. The oedema fluid collected after six hours perfusion at 37·5° C. was devoid of activity whereas that collected after a similar perfusion at 47·8° C. contained the equivalent of 6 μg. adenosine per ml.

We were not able to demonstrate this activity in extracts of thoroughly perfused, minced and ground muscle, skin or connective tissue digested at 48° C. for two hours before boiling and filtering. The muscle extract contained some active adenyl compounds and was rich in inactivating enzyme; the connective tissue extract possessed traces of cardiodepressant activity which were removed by incubation with liver extract. The skin extract contained 1 μg. histamine per ml. and was only tested on the heart preparation in 0·5 ml. dose.

We also examined the effect of digesting for two hours at 48° C. cat serum diluted 1 in 4 with saline, cat red blood cells in a 1 in 40 suspension in saline, the platelets from 20 ml. of cat blood suspended in 8 ml. of saline, citrate plasma almost free from platelets diluted at first with an equal volume of 2 p.c. citrate saline and subsequently 1 in 4 with normal saline and finally a similar dilution of plasma from which the platelets had not been removed. None of these preparations after incubation at 48° C. boiling and filtering exhibited the cardiodepressant activity which we sought. The serum and the platelet suspension were wholly devoid of activity; the suspension of cat red blood cells contained in 1 ml. active adenyl compounds equivalent to 4 to 5 μg. adenosine and its activity was completely removed by incubation with cat liver extract; citrate plasma was active but its activity was exactly matched by a 1 in 8 dilution of 2 p.c. citrate saline.

Inorganic Phosphate.

Our results were obtained from fifteen experiments between 38° C. and 48° C. At the lower temperatures between 37·5° C. and 41° C. there was a small output (20–29 μg. P per min.) in the first hour and in one experiment 37·5° C. this fell gradually to 11 μg. per min. in the fifth hour, rising again to 15 μg. per min. at the end of seven hours. The higher the temperature between 38° C. and 48° C. the earlier was there superimposed upon this initial output a much larger one attributable to tissue injury. There was a sudden increase in output which at 48° C. and 47° C. took place during the first hour, at 45° C. in the third hour, at 43·8° C. to 42·4° C. in the third or fourth hours and at 41° C. to 40° C. in the fourth to the sixth hour. At 39° C. this increase did not occur during nine hours perfusion.

A characteristic feature of a number of these experiments was the occurrence of one or more short periods during which the steady rise to a maximum was interrupted by a transient rise or fall not attributable to changes in rate of flow. For example, in an experiment at 45° C. the values in μg. P and rates of flow in ml. per min. (in brackets) for successive half hour periods during perfusion for six hours were 45 (1·22), 52 (1·00), 56 (1·22), 71 (1·12), 81 (1·12), 154 (1·15), 127 (1·13), 162 (1·30), 158 (1·23), 195 (1·28), 192 (1·35) and 175 (1·22). In other experiments such changes were associated with spontaneous alterations in the rate of flow, e.g. in a perfusion for six hours at 48° C. the successive half hour samples gave the following values, the figures in brackets giving the corresponding rates of flow: 35 (0·35), 101 (1·37), 236 (3·48), 178 (2·08), 180 (1·93), 154 (1·35), 157 (1·37), 179 (1·93), 174 (1·58), 148 (1·37), 128 (1·23), 117 (1·17). The sharp rise in the third sample is attributable to a sudden spontaneous increase in the rate of flow and the apparent maximum at the end of the fourth hour has a similar explanation.

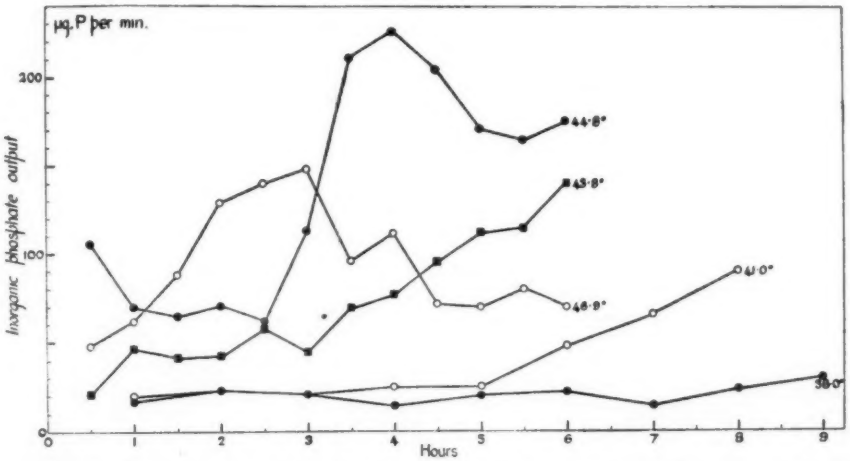

Fig. 3. Output of inorganic phosphate from the cat hind limb. Ordinates, μg. P per minute: abscissae, time in hours.

Fig. 3 shows the output plotted against time in five experiments; irregularities in the graphs like that shown for 46·9° C. between the third and fourth hours are attributable to spontaneous changes in the rate of flow, the rates of flow for the third and fourth hours being unduly high. In the curve for 44·8° C. the high levels reached about the fourth hour are also exaggerated by a spontaneous increase in the rate of flow. The remaining graphs exhibited variation in rates of flow which had no significant effect on the total output.

Alkaline Phosphatase.

Our results were obtained from eleven experiments at temperatures ranging between 38° C. and 48° C. Alkaline phosphatase activity in the samples has been calculated as milli-micrograms of phosphorus capable of being liberated per minute at 36° C. by hydrolysis of sodium β-glycerophosphate (Rawlinson and Kellaway, 1944). We used magnesium as activator in all our experiments and in five the activity was also measured without activation. The results show some individual variation and the experiments are not sufficiently numerous to give a clear cut story

of the effects of temperature particularly in view of the presence of blood in the samples during the early hours of perfusion.

In six experiments 41·0° C. (2), 40·3° C., 39·0° C. and 38·0° C. (2) the output in the first hour was low whereas in five experiments between 42·4° C. and 48·0° C. high values were observed even in the first half hour of perfusion.

In one experiment at 38° C. the values for nine successive hourly samples were 5 (3), 12 (3), 13 (4), 4 (2), 0 (2), 0 (0), 8 (5), 6 (0) and 9 (3), (the figures in brackets being unactivated values). At 39° C. the corresponding values were 9 (9), 6 (5), 7 (6), 5 (7), 6 (7), 4 (1), 0 (0), 0 (0) and 2 (0). A second experiment at 38° C. gave however the following values for eight hours perfusion; 5, 86, 77, 21, 22, 14, 105, 105. An experiment at 41·0° C. gave values for hourly samples of 33, 44, 40, 81, 223, 202, 242 and 276 and very similar results were obtained in another experiment at 40·3° C. but with a maximum of 334 at the sixth hour. A second experiment at 41·0° C. gave figures ranging from 32 (21) in the first hour to 45 (17) in the eighth hour with a maximum of 54 (35) at the fifth hour.

In an experiment at 42·4° C. the values for half hourly samples during perfusion for six hours were 203, 93, 136, 97, 80, 128, 91, 24, 26, 35, 26 and 56. At 45° C. two experiments gave consistently high values during perfusion for six hours; in one of these were 190 (141), 224 (113), 173 (98), 134 (90), 167 (95), 193 (131), 196 (158), 248 (181), 238 (179), 256 (186), 272 (200) and 250 (208). Two experiments at 47° C. and 48° C. gave much lower values than those observed at 45° C. At 48° C. these were 17 (5), 58 (20), 123 (18), 100 (21), 95 (40), 51 (21), 58 (28), 104 (28), 62 (14), 47 (4), 62 (10) and 68 (11).

It appears that at temperatures above 42·4° C. alkaline phosphatase is liberated even in the first half hour of perfusion in considerable amounts though this is less striking at 48° C. Between 38° C. and 41° C. the output in the first hour was relatively low and in two of three experiments below 40° was insignificant throughout.

Lipase.

We determined lipolytic activity in the perfusate in eleven experiments between 38° C. and 50° C. Eight other experiments in which the cabinet alone was raised to a high temperature provided confirmatory evidence in the range between 42° C. and 52° C. Our results are expressed as micro-equivalents of triacetin capable of being hydrolysed per minute at 37° C. by the perfusate; if b ml. N normal sodium hydroxide were required to titrate the acid liberated when c ml. perfusate was incubated at 37° C. for m minutes and if v ml. perfusate flowed in t minutes, then μE per minute is $\frac{Nbv}{cmt}\ 10^3$.

During the first half hour at high temperatures and the first hour at lower temperatures there was generally no significant output of lipase into the perfusate but in four of our eleven experiments in which there was a good deal of blood in the earlier samples the lipolytic activities of the first samples were 0·07, 0·06, 0·03 and 0·03 μE units.

In a single experiment at 50° C. an output did not commence until the beginning of the fifth hour and even then never reached a high level during perfusion lasting six hours, the last four half hour samples having values of 0·03, 0·03, 0·01 and 0·00. At 48° C. in two experiments the output commenced in the first and second hour and continued to the end of perfusion. In one, the values for successive half hour samples were 0·02, 0·10, 0·21, 0·15, 0·15, 0·10, 0·13, 0·09, 0·07, 0·05, 0·11 and 0·11.

At 45° C. the output commenced early in the fourth hour and increased to the end of the sixth hour, the last seven samples in one such experiment being 0·00, 0·03, 0·04, 0·07, 0·12, 0·15 and 0·20. Another experiment at 45° C. illustrates the effect of blood in the early samples, this was considerable in the first two half hourly samples and steadily decreased thereafter; the values for the twelve samples were 0·06, 0·04, 0·07, 0·07, 0·10, 0·09, 0·13, 0·13, 0·13, 0·18, 0·20, and 0·26.

At 41° C. in two experiments significant output first occurred in the fourth and fifth hours and reached maxima of 0·07 and 0·12 respectively in the seventh hour. At 40·3° C. the output first occurred in the fifth hour, the values for hourly samples during perfusion for eight hours being 0·00, 0·00, 0·01, 0·01, 0·03, 0·09, 0·04 and 0·08. The low value for the seventh hour may be attributed to a spontaneous diminution in the rate of flow.

In two experiments at 38° C. the output occurred in the seventh and eighth hour in one and in the ninth hour in the other and then reached a level of only 0·04 μE.

It appears that apart from the lipolytic activity attributable to the presence of blood in the perfusate there is a small but significant output of lipase at temperatures between 41° and 50° C.; this commences in the fourth or fifth hour except in experiments at 48° C. when it occurs during the first hour of perfusion.

Proteolytic Enzymes.

Our results are based upon eight experiments at temperatures between 40·3° C. and 50·0° C. The activity of the samples has been expressed as micro-equivalents of base-titratable groups liberated per ml. perfusate when digested with protein substrate for 40 hours at 36° C. Though

too few in number to demonstrate a time-temperature relationship, our experiments show that above 41° C. significant output of proteolytic enzymes occurs which tends to be greater at the higher temperatures.

At 50° C. with gelatine substrate the following values for half hourly samples were obtained; 10, 12, 13, 13, 13, 4, 19, 0, 0, 0, 3 and 7. At 46·9° C. with myosin substrate the values were 4, 13, 13, 18, 11, 19, 13, 11, 8, 9, 9 and 13. At 41·0° C. with gelatine substrate the values for hourly samples during perfusion for eight hours were 8, 3, 5, 0, 0, 0, 7 and 15 and at 40·0° C. no significant output was observed.

DISCUSSION.

The perfusion experiment cannot afford direct evidence concerning the still vexed question as to whether the systemic effects of burns (apart from bacterial infection) can be wholly explained by haemo-concentration as Underhill and his coworkers (1930) believe or whether they may in part be caused by toxic substances (Wilson *et al.*, 1937, 1938). It may, however, prove to be useful in the search for toxic substances whether these be cell constituents or formed, consequent upon heat injury, in the cells or tissue spaces by enzymatic action.

In perfusion experiments a uniform rate of flow is greatly to be desired, but this is difficult to achieve in the isolated limb. Spontaneous changes in the rate of outflow are liable to occur through opening up or closing down of capillary fields, and this makes it difficult to remove all the blood from the limb during the preliminary perfusion. Diminution of outflow with a constant rate of inflow also occurs during the development of oedema, but as its rate of formation diminishes an increase in outflow is inevitable unless the inflow is controlled.

Our observations on the hydrogen-ion concentration of the perfusate indicate that its increase during prolonged perfusion was greater at 44° to 45° C. than at lower or higher temperatures in the range 37·5° to 50° C. It seems likely that 44° to 45° C. is the optimal region for the acid-producing enzyme systems and that at at higher temperatures some of them are inactivated. The addition of bicarbonate to the perfusion fluid prevents the large increase in acidity which was observed in its absence at 45·5° C., and which must have favoured autolysis (Bradley, 1938). That heat injury can take place independently of autolysis is indicated by experiments at this and higher temperatures in which the pH of the perfusate remained throughout on the alkaline side of neutrality.

We have investigated the liberation of histamine in these perfused organs because it is in general the most suitable pharmacologically active substance as an indicator of the degree of cell injury in perfusion experiments. In experiments on the cat fore limb there was much variation in the amounts liberated with presumably similar degrees of injury; we have not attempted to correlate this with the amounts of histamine in the skin and other tissues. The estimation of histamine is complicated by the presence in the perfusate during the early hours of perfusion of a substance or substances which produce relaxation of the isolated guinea-pig jejunum and antagonize the stimulant effect of histamine. We have no evidence to offer concerning the nature of this activity.

The recent experiments of Green and Bielschowsky and Green (1943) have focussed attention upon the similarity between the systemic effects in traumatic shock and those produced by adenosine triphosphate and upon the possible importance of the pyrophosphate radicle. It should be recalled also, that Bennet and Drury (1931) demonstrated the liberation of pharmacologically active adenyl compounds from cardiac muscle injured by heat. Our results stress the extreme efficiency of the enzyme inactivation of such active compounds because even after the most thorough perfusion and using rapid rates of flow we were unable to detect these substances in the perfusate. In acute injury of the liver by cobra venom, Kellaway and Trethewie (1940) were able to inhibit the enzyme with sodium cyanide and to estimate active adenyl compounds in the perfusate with very little loss by inactivation. They also demonstrated these compounds by collecting the

perfusate at 95° C. The application of these methods to the isolated limb failed to prevent inactivation. This difference in behaviour between the isolated liver and limb, so far as collection at 95° C. is concerned, may depend on the more complicated vascular system of the limb which provides a longer time of exposure at a higher temperature to the action of inactivating enzyme. The failure of cyanide to inactivate even at a concentration of M/40 parallels the observation of Kellaway and Trethewie for the isolated kidney perfused with cobra venom.

There was, however, some cardiodepressant activity in the perfusate from the guinea-pig hind limb at 43° and 45° C. and from the cat fore limb between 42° and 50° C. This was unaffected by cat liver or cat muscle extract rich in enzyme capable of inactivating adenyl compounds. It was also present in oedema fluid from the subcutaneous tissues in heat injury at these temperatures and in traces in that from the guinea-pig hind limb perfused at 37·5° C. It was heat stable and resisted boiling in the presence of acid. Preliminary experiments to discover its source by digesting at 48° C. extracts of perfused muscle, skin, and connective tissue and similar treatment of red blood cells, plasma, serum and platelets from cat blood all yielded negative results. We also failed to produce it by digesting at 48° C. perfusate and oedema fluid obtained from the cat limb at 37·5° C. From these experiments which we are unable at present to extend we cannot offer an opinion either of the nature or significance of this cardiodepressant activity.

The steady leak of inorganic phosphate (20 μg. per minute) during perfusion at 37° to 38° C., which greatly exceeds the amount, which could be derived from the blood still present in the tissues, illustrates the highly abnormal conditions imposed by perfusion with saline in that phosphoric acid which would normally be re-utilized in muscle metabolism is being continually removed. Some phosphate may also come from bone. Our observations take account only of inorganic phosphate, but it seems likely that organic phosphates are also set free. The liberation of the much larger amounts of inorganic phosphate which attended heat injury must be the resultant of a complex series of enzymatic changes favoured by the rise in temperature.

The sources of alkaline phosphatase in the limb as indicated by Bourne (1943) by histological study are bone, vascular endothelium, involuntary muscle, and some of the structures connected with skin. Though our results are exceedingly variable, they indicate that this enzyme is readily liberated even in minor degrees of heat injury and its presence at low temperatures in the perfusate during the early hours of perfusion cannot always be accounted for by the presence of blood. This may possibly be attributable to early liberation from vascular endothelium. The extreme variations in output may well result from individual varations in the content of the tissues.

The demonstration that lipase and proteolytic enzymes were set free from the perfused hind limb of the cat above 41° C. gives colour to the possibility that toxic products may be formed by enzyme activity in the tissue spaces.

SUMMARY.

From the perfused hind limbs of the guinea-pig, histamine was liberated between 45° and 50° C., and histaminase from the uterus in preparations from females. No active adenyl compounds were detected in the perfusate between 37·5° and 53° C., but at 43° and 45° C. there was cardiodepressant activity, more prolonged than that of adenosine, which was not diminished by incubation with cat liver extract. Subcutaneous oedema fluid had similar activity.

From the cat fore limb histamine, but no histaminase, was liberated between 43° and 50° C. In the early hours of perfusion a smooth muscle-relaxing substance which inhibited the stimulant effect of histamine was present in the per-

fusate. No active adenyl compounds could be detected between 37·5° and 50° C., but cardiodepressant activity, similar to that in perfusates from the guinea-pig hind limbs, was found between 42° and 50° C. This was present also in the subcutaneous oedema; it was heat stable and was not diminished by incubation with cat muscle or cat liver extracts.

The hydrogen-ion concentration of the perfusate showed greatest increase at 44° to 45° C. The small output of inorganic phosphates at 37·5° C. to 41° C. had superimposed upon it at higher temperatures, a much larger output. Alkaline phosphatase, lipase and proteolytic enzymes were also set free above 41° C.

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ON THE GROWTH OF INSECT POPULATIONS WITH SUCCESSIVE GENERATIONS

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INTRODUCTION.

Any species of insect tends to increase in numbers and occupy the available places in its environment. When it has done this, the population is said to have reached saturation density. In nature, the number of individuals of a given species, in a given situation, varies from time to time, because changes in the physical and biotic conditions of its environment may favour increase of the population during certain periods, and restrict it during others. One species may reproduce in such a way that, at any given time, the population consists of individuals in all stages of development and belonging to different generations. With another species, the population may be dominated by individuals about the same stage of development, because of incomplete overlapping of the generations. For instance, in cold climates, most insects hibernate, in a resistant stage of development, during the cold months; in warm climates, many species aestivate, in a resistant stage of development, during the hot, dry months; the non-resistant individuals in the population die out during these periods of dormancy. When the dormant period comes to an end, and the insects again become active, the population of any one of these species consists only of individuals about the same age, and in the same stage of development. During the remaining months of the year, which may be referred to as the "growing" period, these insects reproduce through one or more generations. If a species has a short oviposition period, and passes through few generations, the latter may remain more or less distinct, because of the small amount of overlapping of the generations which takes place. If a species has a long oviposition period, or passes through several generations, complete overlapping of the latter may be obtained.

During the past few years, the writer has studied the growth and fluctuations in the density of natural populations of *Smynthurus viridis* L. (Collembola), and *Thrips imaginis* Bagnall (Thysanoptera). These examples illustrate the way in which trends in the growth of insect populations, with incomplete, overlapping generations, are related to those of populations having complete overlapping generations. Thompson (1931) employed certain formulae to calculate populations with organisms reproducing in overlapping generations, under the special conditions "when no causes of mortality intervene."

GROWTH TRENDS IN NATURAL POPULATIONS OF *SMYNTHURUS VIRIDIS* L.

In Fig. 1 are shown trends in the growth of natural populations of *S. viridis* L., in two situations in a clover pasture in Western Australia, during 1936.

The two situations are half a mile apart: they differ in soil type and vegetative cover (Norris, 1938). Each point on the curves represents the number of the insects in an area of 96 square inches. Samples of the population were taken by Norris, twice weekly, in each situation. Three samples were taken each sampling day, the total area of pasture included in three samples being 96 square inches. Mr. Norris, who has discussed his observations in the paper referred to above, kindly sent me the counts of the insects taken each sampling day. The total insects re-

corded, each sampling day, have been smoothed as progressive means of pairs of sampling days, and curves fitted to the data (Fig. 1).

S. viridis lays its eggs on the soil surface usually in one batch of about 40 eggs; the eggs are all laid about the same day, soon after the female becomes adult; but a female may lay a further batch two or three weeks later. In Australia, the species occurs in regions having a winter rainfall: it is active only during the wet season (May to October): it passes through three or more generations a year, according to the length of that season. The insects die out at the end of the wet season, and the species survives the dry season (November to April) as dormant, oversummering eggs, which hatch with the following autumn rains (Davidson, 1937).

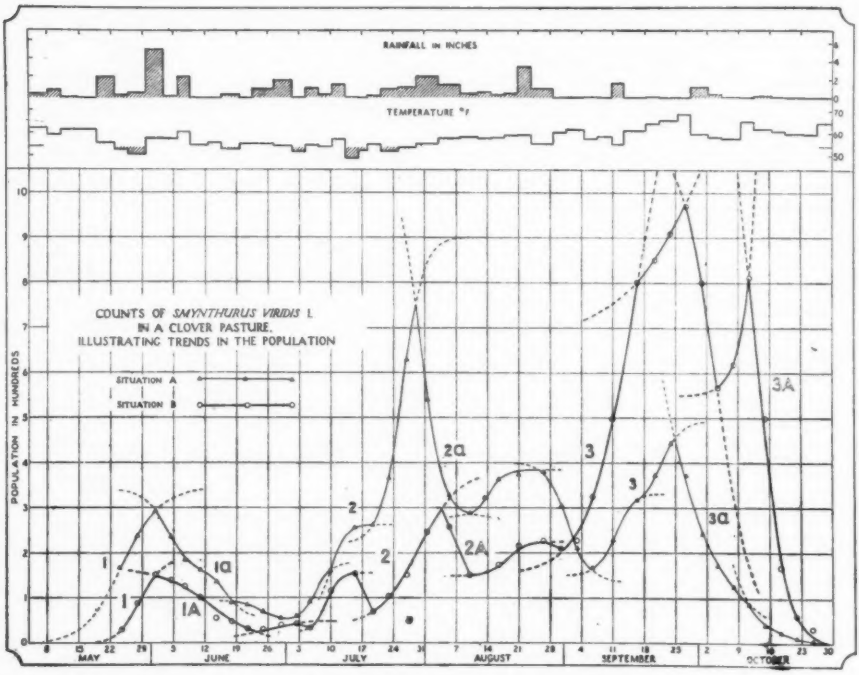

Fig. 1. Calculated curves showing trends in the growth of a natural population of *Smynthurus viridis* L. in two situations in a clover pasture. Each point on the curves represents the number of individuals in an area of 96 square inches of the pasture, on the given date, expressed as progressive means in pairs.

In Fig. 1, the oversummering eggs began to hatch early in May, and the active insects died out towards the end of October. In each situation, the population exhibits three, clearly defined, peak densities, which are mainly due to the appearance of newly hatched nymphs in three successive generations. Each generation exhibits a period of rising population (1-3) and of falling population (1A to 3A). Owing to the heterogeneity of conditions in the pasture, due to changing physical conditions caused by the weather, and by the growth of the plants, some individuals in the population develop more quickly than others, and some are more successful in reproduction, so that the hatching period is longer in the second generation, and, as the wet season advances, some overlapping of the generations occurs. The potential increase of the population, in a generation, will be more or less realized,

according to the degree of favourableness of conditions in the environment. For instance, in situation A, the peak density of the population in generation 3 (about September 25th), is less than would be expected from the magnitude of the peak density in generation 2 (about July 27th) : the potential increase from generation 2 to generation 3 was only partially realized, owing to adverse conditions during the period. Because of the short oviposition period with *S. viridis*, and the small number of generations which can develop in one season, pronounced overlapping of the generations may not occur in nature, under the conditions in the environment of a uniform pasture.

The number of oversummering eggs available for hatching in a given situation in the pasture, at the beginning of the wet season, will vary from year to year, according to the effect of the physical conditions in the pasture, during the previous months, on the reproduction of the insects and survival of the eggs. If the number of oversummering eggs is small, the number of newly-hatched nymphs may be well below the saturation value in the first and second generations; and the density of the population will tend to increase in subsequent generations as in Fig. 1. If the number of eggs is large, the newly-hatched nymphs may temporarily saturate the environment in the first or second generation, and the density of the population will tend to decrease in the following generation. Taking all these matters into consideration, it is evident that fluctuations in the density of the population will vary considerably from year to year in the same area of pasture (Davidson, 1937).

GROWTH OF A THEORETICAL POPULATION OF *SMYNTHURUS VIRIDIS* L.

In Fig. 2(a) is shown the trend in the growth of a theoretical population of *S. viridis* calculated for five generations. The potential rate of reproduction of the species is determined by the following conditions:

1. The sexes occur in equal numbers.
2. The female lays all her eggs, 40 in number, on the first day of her adult life: all the eggs hatch.
3. The eggs commence to hatch 10 days after being laid: they hatch in a normal distribution, over a given period of days.
4. The nymphs become adult 30 days after being hatched.
5. Mortality follows a normal distribution, which allows that all the insects produced in a generation die out by the 45th day after the last eggs of that generation hatch.
6. The environment is constant throughout the period; and the effect of density on the increase of the population is not taken into account, which means that the environment is considered to be unlimited.

The growth of the population extends over a period of 230 days, which may be designated as a wet season, extending from April 1st to November 16th: the insects die out by the 230th day, and the eggs of the 5th generation remain dormant as oversummering eggs.

It is seen from Fig. 2 (a), that the density of the population increases with each successive generation. The potential increase is realized in each generation, because the environment is unlimited, and deaths occur only as a normal distribution, based on the duration of the life span of the insect. In each of the five generations there is a period in which the population curve follows an upward trend, caused by the hatching out of nymphs of the particular generation; and a period during which the curve follows a downward trend, because deaths exceed births: the latter period becomes progressively less in each successive generation, owing to overlapping of the generations. The first generation consists of 40 nymphs, which hatched out from 40 oversummering eggs, during the first five days (Table 1). Therefore, the growth of the population during this period can be represented by a cumulative, normal dis-

tribution curve, which attains the maximum density of 40 nymphs on the fifth day, deaths during the hatching period being negligible. All the individuals in generation 1 die out by the 50th day (that is, 45 days after the last oversummering eggs hatched). Therefore, the decline of the population during the period 5 to 50 days, can be represented by a normal survival curve, commencing at 2.5 days and ending on the 50th day, the 40 nymphs being considered as all of the same age. On the

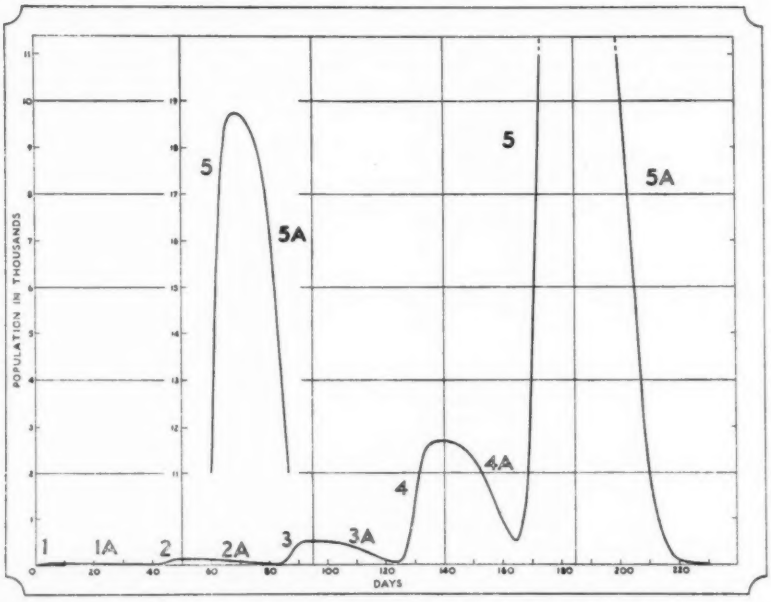

Fig. 2 (a). A calculated curve showing the growth of a theoretical population of *S. viridis* through 5 generations, when the environmental conditions remain constant and births and deaths in each generation are normally distributed. Values on the "y" axis do not include eggs. The top of curve 5 has been inset to save space.

40th day, the population begins to increase again owing to the hatching out of nymphs of the second generation: in subsequent generations a similar upward trend in the population begins about the 80th, 120th, and 160th day, as a result of the appearance of newly-hatched nymphs in successive generations.

TABLE 1.

The essential data relating to generations 1 to 5 in Fig. 2 (a).

Generation.	Hatching period (days).	No. of nymphs hatched.	End of generation. Day.	Date.	No. of adults (based on mid-date).
1	1– 5	40	50	May 20	5·6
2	40– 50	112	95	July 4	24
3	80– 95	480	140	Aug. 18	134
4	120–140	2,680	185	Oct. 2	944
5	160–185	18,880	230	Nov. 16	10,788

The methods employed in constructing Fig. 2 (a) are demonstrated by reference to curves for the period of the 3rd generation (80 to 140 days), shown in Fig. 2 (b). The calculated number

of nymphs hatching out in the 3rd generation is 480: they hatched out in a normal distribution, over the period 80 to 95 days (A): if all the nymphs survived to the 95th day, there would be a total of 480 nymphs alive on that day (C). The distribution of deaths of these insects is presented as a normal distribution curve, all the insects being dead by the 140th day, that is 45 days after the last hatching for eggs of the 3rd generation. If 480 nymphs are present on the 95th day, they will vary in age from 1 to 15 days. Therefore, the distribution of the survivors, for this generation, cannot be represented as a simple, survival curve extending from the 95th to the 140th day, because, not only are the nymphs not of the same age, but some of them may die during the hatching period (80 to 95 days). It would be appropriate to sub-divide the hatching period into smaller units, say 6 units of 2·5 days each, the nymphs which hatched out during each unit period of 2·5 days, being considered as of the same age: a compound, survival curve could be then constructed from the series of 6, overlapping, survival curves. Calculations show that, in the example under consideration, few of the nymphs die during the period 80 to 95 days, so that a normal distribution curve showing mortality was constructed, commencing at the mid-date of the hatching period (87·5 days), and ending on the 104th day (D): for this purpose, the 480 nymphs were considered as being of the same age. For any day in the period 80 to 95 days, the number of insects alive is obtained by deducting the number of nymphs dead from the number hatched. In the case of Fig. 3, the computations show that the number of nymphs which die during this period is negligible. For any day in the period 95 to 140 days, the number of insects alive is obtained by deducting the cumulative total insects dead by the given date, from the maximum number of nymphs which hatched in the generation, that is 480 in generation 3. The numbers of insects carried over from generations 2 and 4, due to overlapping of the generations, are added to those of generation 3, on the appropriate days. The curves C and E in Fig. 2 (b) represent the curves 3 and 3A in Fig. 2 (a).

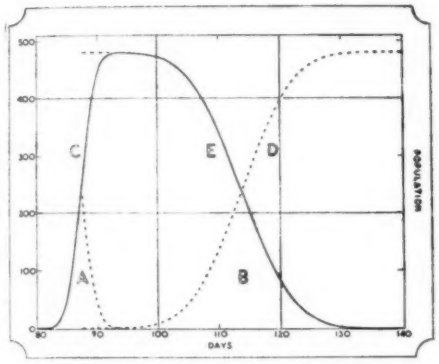

Fig. 2 (b). Showing the computed normal distribution curve for hatching (A) and deaths (B) of *S. viridis* in generation 3, also the cumulative hatching curve (C) and the cumulative mortality curve (D), in order to illustrate the method used in constructing Fig. 2 (a).

The number of adults, which mature in each generation, can be calculated from the population curve (Fig. 2 (a)). For example, in generation 3, the nymphs become adult during the period 110 to 125 days, and the number of adults can be calculated from Fig. 2 (b). In order to reduce the amount of arithmetic, the number of adults produced in generation 3 is taken as the number of insects alive on the mid-date of this period, that is 134 on the 117·5 day. 134 adults give 67 females, which produce 2,680 eggs, so that 2,680 nymphs hatch out in the 4th generation.

OVERLAPPING GENERATIONS.

In Fig. 2 (a) each generation overlaps the preceding one. The second generation overlaps the first by 10 days (40 to 50 days); and the period of overlap increases by 5 days, in each successive generation, so that the fifth generation overlaps the fourth by 25 days (160-185 days). If the growth of the population were continued to the ninth generation, the first day of hatching in that generation would coincide with the last day of hatching of the eighth generation (320th day). When complete overlapping of the generations is attained, the decline of the population in each generation, which obtains in Fig. 2 (a), tends to be eliminated.

If the hatching period in the first generation were 10 days instead of 5, the second generation would overlap the first by 20 days; and the first day of hatching in the fifth generation would coincide with the last day of hatching in the fourth generation (145th day). This implies that the number of generations which must be passed through, before complete overlapping is attained, varies with the species: the number is greater with those species which lay their eggs during a relatively short period of their adult life.

With species in which the female lays eggs at frequent intervals throughout her adult life, a well-mixed population, of individuals of all ages, can develop early in the growth of the population; and the generation rise and fall in population density, seen in Fig. 1, may be practically eliminated. This is seen in Fig 3, which represents the growth of a natural population of *Thrips imaginis* L., during September to December, 1933, as represented by the number of adults present in roses, in the Waite Institute garden, during this period. The points on the curve show the average number of adults per rose, per day, based on daily counts of the adults present in 20 roses: the daily averages were smoothed, by taking progressive daily means, in 10-day periods, from August 1st to December 22nd.

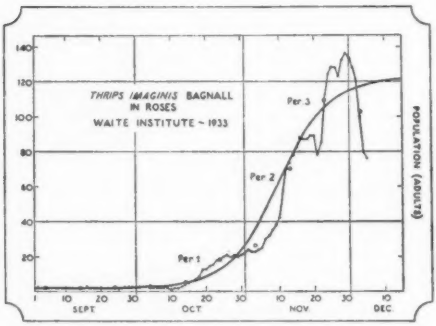

Fig. 3. Showing trend in the growth of a natural population of *Thrips imaginis* L. (adults only) in roses. The points on the curve represent the average number of adults per rose per day, expressed as progressive daily means for 10-day periods. The calculated curve illustrates the general trend of the population.

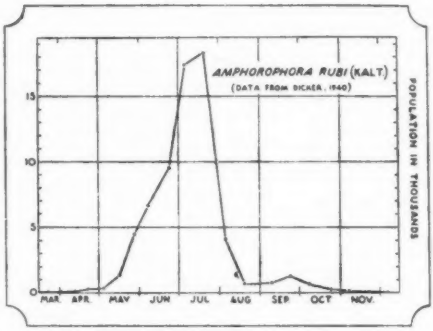

Fig. 4. Showing trend in the growth of a natural population of *Amphorophora rubi* (Kalt.) on raspberry canes in England: the points on the curve represent the total number of individuals on 100 raspberry canes on the given dates.

Thrips imaginis is a flower-inhabiting species whose females can lay eggs almost daily throughout adult life. In the Adelaide district, the species is active throughout the year. The population begins to increase in September, and the increase is sustained throughout the spring and early summer (Fig. 3). The population declines from December onwards, owing to the hot, dry, summer months; and the density keeps at a relatively low level until the following spring.

These features of the population are present every year; but the maximum density for the year, and the date when it exists, varies from year to year, as is shown in the population graphs for the years 1932–36 (Davidson, 1936).

Compared with *S. viridis* (Figs. 1 and 2 (a)), the generation rise and fall in the density of the population of *T. imaginis*, is almost eliminated (Fig. 3). The general trend in the growth of the population, during September to the end of November, is demonstrated by a logistic curve having the formula:

$$y = 2 + \frac{120}{1 + e^{14{\cdot}04586 - {\cdot}138156x}}$$

The formula was calculated from values for the average, progressive, daily mean, for each of the 10-day periods from September 1st to November 28th. These nine values are plotted as circles in Fig. 3. The root-mean-square deviation for the nine observed and calculated values is 4·29. The curve demonstrates that the general growth trend of populations of *T. imaginis* resembles that obtained by Pearl (1927) and Bodenheimer (1938) with laboratory cultures of *Drosophila*.

The progressive daily means plotted in Fig. 3 indicate that the growth of the population during October and November proceeds in three cyclic periods. These secondary trends are related to the appearance in the roses of adults of the second and third spring generations, and to the influence of the prevailing spring weather on the rate of development of the insects, and on the movements of the adults. The course of these secondary trends varies from year to year; and the cyclic character exhibited in 1933 is not apparent every year. The rise in the adult population in roses, which begins in October in 1933, appears in September or October every year. It is mainly caused by the migration into roses of adults from spring flowers, such as *Echium plantagineum* (Evans, 1933). The temporary drop in the population about November 20th, 1933, is associated with cool weather, which reduced the activity of the adults, so that fewer individuals visited the roses during the period.

A similar trend in the growth of a natural insect population is seen in the case of the aphid *Amphorophora rubi* (Kalt), which lives on varieties of *Rubus*, such as raspberry and blackberry. On the raspberry, sexual females lay eggs about the middle of October, on the canes and beneath the leaves. The overwintering eggs hatch out the following spring, and the species produces several parthenogenetic generations during the summer: sexual females are produced in autumn; overwintering eggs are laid on the plants; and the active insects die out before the end of the year.

The growth of a population of *A. rubi*, on raspberry canes in England during 1938, is illustrated in Fig. 4. The points on the curve represent the number of individuals counted on 100 canes, at approximately fortnightly intervals: the raspberry plantation was divided into 5 equal blocks, from each of which 20 canes were selected for making the aphid counts, so that the counts represent the growth of the aphid population in the plantation. The data are taken from the work of Dicker (1940). In the year represented, the overwintering eggs were hatching early in March. Some individuals of this first generation were adult early in April, and produced young individuals of the 2nd generation. Adults of the 3rd generation began to mature during the first week in June, and produce young of the 4th generation (Dicker, 1940).

Owing to the long intervals between sampling days, the published data are inadequate for the purpose of calculating the detailed trend in the growth of the population. However, the general trend is evidently the same as in the case of *T. imaginis*, and can be represented by a logistic type of curve. The population declined rapidly in late summer, and eventually the active insects died out in early December. The sustained decline of the population in late summer is a feature of aphis populations in England, which appears to be caused by a combination of weather, parasities and predators, and seasonal condition of the host plant.

UNLIMITED AND LIMITED INCREASE OF AN INSECT POPULATION.

In Fig. 5 three curves are shown, which illustrate trends in the growth of three theoretical populations of *S. viridis*, when the environment is considered to be unlimited or restricted. The species has the potential power of increase as defined on page 97. Each curve represents the trend in the increase in the number of insects in successive generations, under the given conditions: it does not show the actual course of the growth of the populations during the period of each generation, as in Fig. 2 (a).

Curve A represents unlimited growth of the population when there are no deaths: the potential increase of the insect is fully realized. The values for y at 5, 50 and 95 days, represent the peak density in each of the first three generations. The formula of the curve is: $y = ab^x$ where x = number of the generation: y = maximum density of the population in that generation: a = 2: b = 20.

Curve B represents unlimited growth of the population, when allowance is made, in each generation, for a normal distribution of deaths, in accordance with the life span of the insect; and for a normal distribution of the hatching out of nymphs, in each generation. The values for y at 5, 50, 95 and 140 days, represent the maximum density of the population in each of the first

4 generations (see Fig. 2 (a)). The potential increase of the population is realized in each successive generation.[1]

Curve C represents the restricted rate of growth of a population which saturates its environment, when the density reaches 20,000 individuals. It is a logistic curve, which was calculated on the basis that the values for maximum density of the population, in the first two generations, namely 40 individuals on the 5th day, and 112 on the 50th day, lie on the calculated curve.

The formula for the curve is:

$$y = \frac{20{,}000}{1 + e^{6{\cdot}3001 - {\cdot}0223x}}$$

where y = population, x = number of days. Compared with Curve B, the rate of increase of the population is lower; for instance, the computed value for "y" on the 104th day is 2,680 in curve B, whereas in curve C it is only 800.

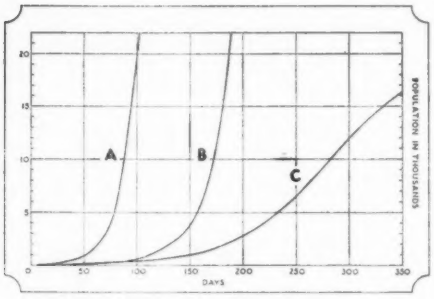

Fig. 5. Three calculated curves illustrating unlimited and limited increase in growth with theoretical populations of an insect. Curve A represents unlimited growth when there are no deaths. Curve B represents unlimited growth when births and deaths in each generation are normally distributed. Curve C represents limited increase in which density restricts the rate of growth, the environment being saturated by 20,000 individuals.

In constructing curve C, it has been assumed that the maximum population, in each generation, falls on the curve. With natural populations in which the generations are distinct as in Fig. 2 (a), this may not happen, because the nymphs, of each generation, will hatch out and die, in a normal or skew distribution, so that the peak density in each of the early generations will obtain only for a short time. However, the *general* trend of the growth of the population, until saturation is attained, will follow the course of a logistic curve if the conditions in the environment remain favourable.

SUMMARY.

Growth trends in natural populations of *Smynthurus viridis* L. (Collembola) and *Thrips imaginis* Bagnal (Thysanoptera) are discussed, in order to show the relation between the growth of insect populations with incomplete overlapping generations, and those species in which complete overlapping of the generations occurs. With the latter group, trends in the growth of the population are best represented by a logistic curve, which was used by Pearl (1927) and Bodenheimer (1938) to describe the course of growth in laboratory cultures of *Drosophila*: in some instances the growth trends may be cyclic, because of generation effects, or change of conditions in the environment. With the former group the growth of the population exhibits a distinctive upward and downward trend in each generation; and the course of growth can be represented by combinations of normal or skew distribution curves, which take into account the distribution of births and deaths

[1] In Fig. 2 (a) the maximum density of the population, in each of the first 4 generations, coincides closely with the total number of nymphs which hatch out in the respective generation, because the number of deaths during the hatching periods is negligible. However, by the 5th generation, the hatching period has increased to 25 days (160 to 185 days): during this period 200 of the newly-hatched nymphs of the 5th generation die, so that the actual number of nymphs alive on the last day of the hatching period is 18,880 − 200 = 18,680. It follows from this that, as the length of the hatching period increases, the number of nymphs which die during this period also increases. Therefore, in a given generation, the maximum number of nymphs alive in that generation may occur earlier than the last day of the hatching period.

in each generation. The extent of the overlapping of generations enlarges as the number of consecutive generations increases; complete overlapping is achieved in fewer generations with those species in which the female lays its eggs, at frequent intervals, over a large part of its adult life. The *general* trend of the growth of an insect population with overlapping generations can be described by a logistic curve: but it may not show the trend of the population within the periods of individual generations.

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A STUDY OF THE IMMUNOLOGICAL REACTIONS OF THE VARIOLA AND VACCINIA VIRUSES GROWN IN THE DEVELOPING EGG

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The transformation of variola virus to vaccinia virus has for many years been the subject of investigation and controversy. Whilst some workers have reported that such variation has been brought about by animal passage, others, particularly French observers, deny that such a change ever takes place experimentally.

Horgan (1938) reported a successful attempt to bring about the variation of variola to vaccinia. As a result of his own experience and an extensive review of the literature, Horgan drew certain conclusions, amongst which were the following:

"(1) There is general agreement with regard to the difficulty of producing lesions in animals, other than the monkey, with human smallpox or alastrim. (2) The assistance afforded by passage through a monkey towards securing subsequent transfer to other animals is generally admitted. (3) Evidence is lacking that continued passage in the monkey can ever bring about a vaccinal variation; the infection seems to remain variola indefinitely."

Evidence so far available indicates that variola virus grown on the chorioallantois of the developing chick embryo, in one respect at least, resembles variola in the monkey; it appears to remain variola indefinitely. The appearance of the foci themselves remains constant over many generations (Lazarus, Eddie and Meyer, 1937; Budingh, 1938; North, Broben and Mengoni, 1944), whilst Nelson (1943) found that variola virus, after repeated egg passage, showed no significant alteration in the species identity on attempted animal inoculation. Torres and Teixeira (1935) infected monkeys with alastrim. From this source they grew alastrim in the egg and after 11 passages infected other monkeys producing typical lesions of alastrim.

The propagation of the variola virus on the chorioallantois affords opportunities, previously lacking, for the study of cross immunity between smallpox and vaccinia. Bohls and Irons (1942) report that by the use of hyperimmune antivaccinal rabbit serum with variola virus they were usually able to obtain a definite reduction in pock counts which could be attributed to the influence of antiserum. The agglutination of fowl red cells by virus preparations (Hirst, 1941; McLelland and Hare, 1941) and its inhibition by specific antisera (Hirst, 1942) has been applied to the titration of vaccinia virus and its immune serum by Nagler (1942). Its application to the study of the relation between variola and vaccinia viruses is reported in this paper.

MATERIALS AND METHODS.

Source of Viruses.

The variola virus was isolated on the chorioallantois from old dried smallpox crusts as previously described (North, Broben and Mengoni, 1944). Suspensions of passages up to the 16th were used. Two strains of vaccinia virus were used. One was grown from stock vaccine calf lymph; the other from a lesion in a human being (F) accidentally infected with vaccinia (Broben and North, 1943). Suspensions prepared from these strains agglutinated adult fowl red cells equally well.

Preparation of Virus Suspensions for Agglutination Test.

The method described by Nagler (1942) was followed with some slight modifications. This consisted essentially of grinding, in a mortar together with silica sand, the virus infected chorio-allantoic membrane, adding 0·5 ml. of suspending fluid per membrane, and centrifuging. The decanted supernatant, tested for bacterial contaminants, constituted a "Standard Suspension".

The vaccinal membranes were collected after two days' incubation. Some suspensions of variola virus were prepared from membranes after two days' growth, others from membranes after three days' growth. As a routine the suspending fluid used was 50 p.c. glycerol in distilled water. As will be described later, some modifications were tried in the case of variola.

Sera.

(a) *Human.* Samples were obtained from two coloured seamen, E.K. and G.V., convalescing from mild attacks of smallpox. They had been vaccinated at some time previously but the detailed vaccination history was lacking. Unfortunately no serum was available from an un-vaccinated convalescent from smallpox. Serum was also obtained from a young man (M) who had not been vaccinated, and from myself (E.A.N.). I have been vaccinated several times and had given an immunity reaction a few weeks previously.

(b) *Animal.* Antivaccinal calf serum was obtained from animals bled 7 weeks after having been inoculated for the production of stock calf lymph. Serum collected prior to inoculation was used as a control.

Fowl Red Blood Corpuscles for Hirst Reaction.

These were obtained and prepared as by Nagler (1942).

Methods of Titration.

The virus titrations were carried out as described by Nagler (1942). Briefly, 0·25 ml. of falling dilutions of virus suspensions were distributed to small tubes and an equal volume of 2 p.c. suspension of red cells was added, followed by 0·25 ml. of saline. After shaking, the mixture was allowed to stand in the incubator at 35° C. for 1 hour and the result was then read. The results were graded +++, ++, +, ± and 0: +++ represented maximal agglutination, etc. A control vaccinia virus titration was always carried out in parallel with any variola virus titrations.

Titrations of agglutination-inhibiting substances in sera (Hirst antibodies) were carried out by:

(a) Using serial virus dilutions and a uniform concentration of serum (Nagler, 1942). To the tubes containing serial virus dilutions, an equal volume of a uniform concentration of serum was added, and after shaking, the tubes were placed in the incubator for 30 minutes. The red cells were then added, and after shaking, the mixture was returned to the incubator for a further 60 minutes before reading.

(b) Using a fixed predetermined concentration of virus and serial dilutions of serum. The virus titration was first made and the tube showing a "one plus" (+) agglutination noted. Virus suspension 5 times the concentration of that originally in the "one plus" tube was then placed in tubes with an equal volume of serial serum dilutions. The remainder of the procedure was as in (a).

EXPERIMENTAL.

Observations on Agglutination of Chick Embryo Red Blood Cells by Variola Virus.

On two occasions during the collection of chorioallantoic membranes infected with variola virus, agglutination of chick-embryo red cells from ruptured vessels was observed. As shown in Table 1 the occurrence was not related to the age of embryo or stage of growth of virus.

A similar phenomenon has not been observed by me in eggs inoculated with vaccinia virus, and this is in accord with the findings of Clark and Nagler (1943).

TABLE 1.

Details relating to spontaneous agglutination of chick embryo red cells in eggs seeded with variola virus.

Serial No. of egg passage.	Proportion of eggs showing phenomenon.	Day of development of embryo when seeded.	No. of days seeded with variola virus.
6th	1/6	10	3
16th	2/8	12	2

Seeding of the allantoic cavity with virus suspensions was carried out using 0·05 ml. of a 1: 20 dilution of a standard virus suspension. After 48 hours' incubation 0·05 ml. of the infected allantoic fluid was used for chorioallantoic inoculation of other eggs. Semi-confluent pocks resulted. This suggested that little, if any, growth occurred in the allantoic sac.

Agglutination of the embryo's own red cells was not seen during the opening of the infected allantoic sacs into which bleeding occurred, but it took place gradually in test tubes to which the blood-contaminated fluids were transferred.

Comparison of the agglutinating power of variola-virus suspensions for embryo and adult fowl red cells was made. Cells from six embryos, ranging from 14 to 17 days development, were tested. Whilst some agglutination occurred with all samples, only one lot was agglutinated to an extent comparable with susceptible adult red cells.

Comparison of Agglutinating Power of Variola and Vaccinia Virus Suspensions for Adult Fowl Red Cells.

From the first attempt to apply Hirst's phenomenon to variola, it became evident that this virus would not behave similarly to vaccinia. Only very heavy suspensions were found to agglutinate the red cells, and the titre was always lower than that of vaccinia. This tended to vary from one generation of egg virus to another, but the variation appeared to be quite inconsistent. A comparison of the Hirst titre of suspensions made from the 2nd to the 13th egg passage virus revealed no steady upward or downward trend in titre. Attempts were made to increase the agglutinating power of the virus by varying (1) the nature of the suspending fluid, (2) the temperature, (3) the red cells (cells from fowls agglutinated poorly by vaccinia virus), but without success. The protocol of a titration showing the effect of suspending the variola virus in (a) saline and (b) 10 p.c. serum saline in place of the routine 50 p.c. glycerol in water is shown in Table 2. It will be seen that in this instance the titre of the variola suspension was ¼ that of the control vaccinia suspension.

The lower Hirst titre of variola as compared with vaccinia was not due to a lesser concentration of viable virus. Titrations of suspensions of the two viruses were carried out in duplicate on several occasions, by the pock-counting technique on the chorioallantois (Keogh, 1936). The titre was substantially the same, round about 10^8. If anything, the variola counts were slightly higher.

TABLE 2.

Comparison of agglutinating power for adult fowl red cells of variola and vaccinia suspensions.

Virus.	Suspending fluid.	Original virus dilutions (final dilutions 3 times greater). 1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	C
Vaccinia	50 p.c. Glycerol water			+++	+++	+++	+++	+	±	−
Variola 13th egg passage	Saline	++	+	±	0	0	0			
do.	Serum saline	+++	++	++	++	+	±			
do.	50 p.c. Glycerol water	+++	++	++	++	+	±			

The notations +++, ++, +, ±, 0 indicate the degree of agglutination as used by Nagler (1942), e.g. +++ = complete agglutination and 0 = no agglutination. Test read after 1 hour at 36° C.

Some improvement in Hirst titre appeared to result from methods aimed at exsanguinating the membranes prior to collection. Unfortunately time did not permit this aspect to be investigated sufficiently for definite conclusions to be drawn.

The difference in actual titre, and the apparent variations in agglutinating power from generation to generation were not the only distinguishing features of red cell agglutination of variola as compared with that of vaccinia virus. The appearance of the masses of agglutinated cells at the bottom and sides of the tubes was somewhat different. The cells appeared to be distributed somewhat more evenly especially at the periphery of the agglutinated material up the sides of the tube.

The deposit of red cells was quite stable. If, after the reaction was complete, the cells were agitated, typical agglutination again occurred. It was found that normal sera (human and bovine) in high concentration did not inhibit the reaction.

Cross Immunity Reactions between Variola and Vaccinia.

Preliminary tests established the fact that anti-vaccinal animal sera inhibited the Hirst reaction with variola virus suspensions. It was also found that the serum of the two smallpox convalescents, G.V. and E.K., did the same with vaccinia virus suspensions. These convalescents had been vaccinated at some indefinite time prior to contracting smallpox, and their sera therefore might be presumed to contain an unknown amount of vaccinia antibody. However, my own serum (E.A.N.) in a concentration of 1: 10 showed a comparatively small inhibitory action against the vaccinia virus and none against variola in the Hirst reaction (see Tables 3 and 4).

As I had given a complete immunity reaction to vaccination a few weeks previously, it seemed unlikely that the smallpox convalescent sera contained more vaccinal antibody than my serum (E.A.N.) even allowing for the possibility of an anamnestic reaction. One of these sera (G.V.) showed a higher Hirst antibody titre against either virus than the other (E.K.) and was therefore used in subsequent immunity tests.

TABLE 3.

Inhibition of Hirst reaction, using fixed predetermined concentration of virus, by variola and vaccinia anti-sera.

Serum.	Dilution of serum.	Variola suspension.	Vaccinia suspension.	Control.
Nil (saline only)		+++	+++	0
Normal bovine	1: 10	+++	+++	0
Anti-vaccinal bovine	1: 50	±	0	
do.	1: 100	++	0	
do.	1: 200	+++	+	
Normal human (M)	1: 10	+++	+++	
Anti-vaccinal human (E.A.N.)	1: 10	+++	++	
do.	1: 20	+++	+++	
Convalescent variola patient (G.V.)	1: 50	0	0	
do.	1: 100	±	0	
do.	1: 200	+	0	
do.	1: 500	+++	++	

The difference in the Hirst titres of the vaccinia and variola virus suspensions made it difficult to judge the relative effect of one antiserum against each of the two viruses.

Findings from representative experiments are shown in Tables 3 and 4. All the variola suspensions for the cross immunity tests (except the preliminary ones) were prepared from later egg passage material: 14th to 16th generations.

TABLE 4.

Inhibition of Hirst reaction, using serial dilutions of virus suspensions, by human variola and vaccinia anti-sera.

	Diln. of		Original dilution of virus suspension.								
Serum (human).	serum.	Virus.	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	C
Nil (saline only)		Variola	+++	+++	++	+	0				0
Normal (M)	1: 10	do.	+++	+++	++	+	0				
Vaccinated (E.A.N.)	1: 10	do.	+++	+++	++	+	0				
Variola convalescent (G.V.)	1: 10	do.	±	0	0	0	0				
do.	1: 100	do.	+	±	0	0	0				
Nil (saline only)		Vaccinia	+++	+++	+++	+++	+++	++	+	0	0
Normal (M)	1: 10	do.	+++	+++	+++	+++	+++	++	+	0	
Vaccinated (E.A.N.)	1: 10	do.	+++	+++	+++	+++	+	0	0	0	
Variola convalescent (G.V.)	1: 10	do.	++	+	0	0	0	0	0	0	
do.	1: 100	do.	+++	++	±	0	0	0	0	0	

It was not possible to make a completely satisfactory comparison of the effect of an inhibiting substance on two mixtures which in any case do not behave quite similarly. Nevertheless an analysis of the results suggested that the variola antisera were more potent against vaccinia suspensions than were vaccinia antisera against variola suspensions.

The effect of anti-vaccinal immune serum on the number of foci developing on the chorio-allantois was only briefly examined. It reduced the number of variola lesions as reported by Bohls and Irons (1942) but this action did not appear as strong as its effect against vaccinia virus.

DISCUSSION.

Owing to circumstances arising from the war situation, it was unfortunately impossible to devote further time to this study. Many interesting questions, therefore, remain either incompletely investigated or wholly unanswered.

It seemed reasonable to anticipate that continued passage in the egg might facilitate the transformation of variola to vaccinia on subsequent animal inoculation; or that the actual transformation would occur in the egg itself. Previous work, however, did not lend support to this expectation. Since the data on which this paper is based were obtained, Nelson (1943) has reported that a strain of variola virus (Minnesota), after being passaged 180 times in embryonated eggs, showed no apparent change in its behaviour. Subsequent attempts to maintain this strain in the rabbit by serial testicular passage were unsuccessful.

The results now reported tend to confirm the earlier reports based on appearance of lesions and the results of attempts at further animal passage. The ability of variola virus suspensions to agglutinate chick embryo red cells, and the continued difference in the Hirst reaction with adult fowl red cells between variola and vaccinia suspensions point to a continued distinction of the viruses on repeated egg passage.

On the other hand the immunological relations as far as Hirst phenomenon and pock-inhibiting antibodies are concerned are at least extremely close; they may be identical. If any difference exists it would appear that the viruses contain common group antigens and that the variola virus has a specific antigen not contained in the vaccinia virus.

SUMMARY.

The present state of knowledge concerning the transformation of variola to vaccinia is discussed.

Previous reports dealing with the appearance of membranes and attempts at animal passage do not suggest that variola is transformed to vaccinia on continued egg passage.

The characteristics are reported of the Hirst reaction using variola virus grown on the egg.

These are different from the Hirst reaction with vaccinia and indicate a difference in the viruses.

The Hirst and pock-inhibiting antibodies of the two viruses are similar, if not identical.

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THE METABOLISM OF STARVING LEAVES

5. CHANGES IN AMOUNTS OF SOME AMINO ACIDS DURING STARVATION OF GRASS LEAVES; AND THEIR BEARING ON THE NATURE OF THE RELATIONSHIP BETWEEN PROTEINS AND AMINO ACIDS.

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Petrie and Wood (1938a and b) showed that during synthesis of proteins in leaves of grass plants the curve relating the concentrations of proteins and of total residual amino acids in the cells was concave to the amino acid concentration axis. Residual amino acids were measured by subtracting the amide-nitrogen contributed by glutamine and asparagine from the total amino-nitrogen determined after precipitation of the proteins. It was concluded that when the amino acid concentration of the leaves increased then one or more of the amino acids was deaminated more rapidly than others and it was shown that one amino acid, viz. cystine, does not always increase when the amount of the total amino acids increases. The resulting disproportionality among the free amino acids would account, at least in part, for the observed relations between proteins and amino acids.

Wood (1942) in an experiment performed under conditions such that protein synthesis occurred concluded that the relationships between respiration rate, amino acids and protein could be best explained by the assumption that some amino acids are deaminated more readily than others.

Phillis and Mason (1942) estimated amounts of protein and of "crystalloid" nitrogen at intervals during the life cycle of cotton plants; they found a relationship between proteins and "crystalloid-N" similar to that described by Petrie and Wood (1938a) between proteins and amino acids, i.e. the curve relating the two variables was concave to the crystalloid-N axis. They pointed out that this relationship was characteristic of apolar adsorption and think the latter to be the most likely explanation of their results.

Wood and Barrien (1939) investigated protein metabolism during starvation of leaves attached to the plant; they found that protein-N in the leaves decreased in amount and was accompanied by a corresponding increase in amount of soluble nitrogen compounds; and also that the protein-sulphur decreased in amount and was accompanied by an equivalent increase in amount of sulphate-S, but that neutral-sulphur compounds, including cystine, did not increase in amount.

In the metabolism of detached leaves during starvation, protein hydrolysis is accompanied first by increase in amount of residual amino acids which reaches a maximum value and then decreases. The decrease is associated especially with the formation of the amides glutamine and asparagine, and also with the formation of ammonia. The amides are derived from a carbon source, probably α-ketoglutaric and oxalacetic acids, and ammonia derived from oxidation of amino acids or by transamination of amino acids and keto acids (Vickery *et al.*, 1939; Chibnall, 1939; Wood, Cruickshank and Kuchel, 1943). Decreased amounts of protein could still exhibit balanced relations with free amino acids under these conditions if some amino acids were utilized more rapidly than others.

In the present paper we have determined the amounts of some individual amino acids, which can be determined in small amounts with a reasonable degree of accuracy, in leaves at different stages of starvation and in both air and nitrogen; also in the light of the knowledge so obtained an explanation is put forward to account for the observed relations between proteins, amino acids and respiration rate.

EXPERIMENTAL.

Method.

Material consisted of leaves of Kikuyu grass (*Pennisetum clandestinum*, Hockst.) grown in a plot in the grounds of the Botany School and of Algerian oats (*Avena sterilis*, L.) cultured in pots under standard conditions in a glasshouse. The experimental procedure was the same in all cases: the plants were cut and the leaves quickly severed at the ligule by a team of workers and the leaves thoroughly mixed and sampled; aliquots were placed in black containers in water baths at 24·50 ± 0·02° C. and streams of air or of nitrogen passed over the leaves during the experimental period at the rate of 4 litres per hour. 20 gm. samples were used for determination

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of respiration rate and 40 gm. samples for analytical material at each harvest. Samples were withdrawn for analysis usually at intervals of 24 hours.

Description of Experiments.

Experiment XI. Leaves of Kikuyu grass were used. Experiment commenced 4–5–43 and the leaves were kept continuously in an air stream for 144 hours. Leaves were full yellow in colour on the 4th day of starvation. Samples were withdrawn for analysis at 24 hour intervals. Measurements of respiration rate were not made. Results of analysis are given in Fig. 1.

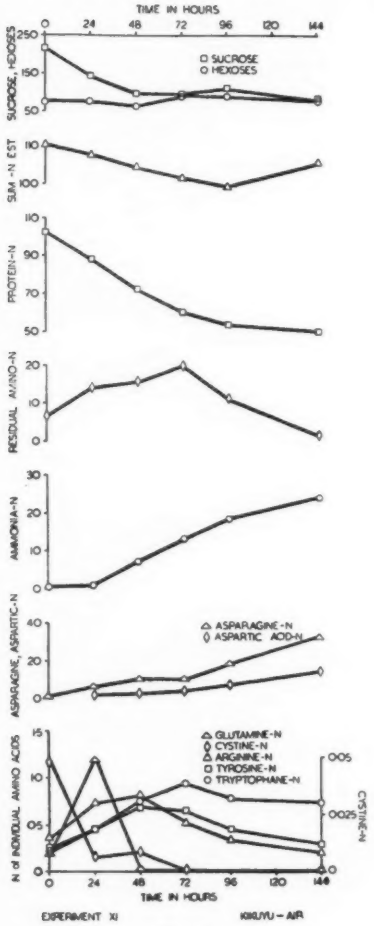

Fig. 1.

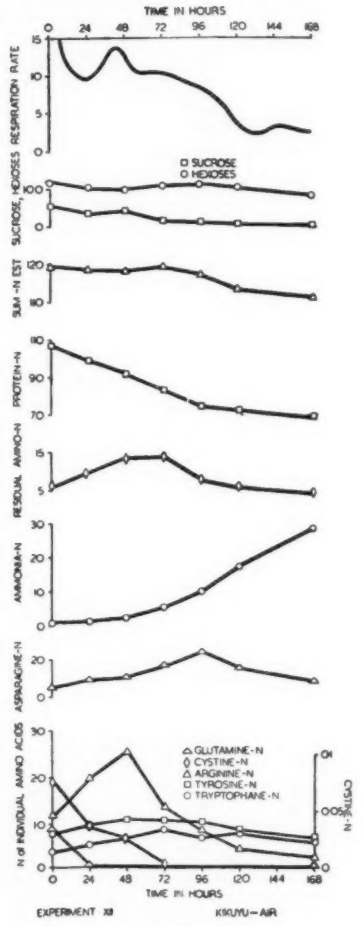

Fig. 2.

Experiment XII. Leaves of Kikuyu grass were used. Experiment commenced 17–5–43 and the leaves were kept continuously in an air stream for 168 hours. Leaves were full yellow on the 4th day of starvation. Samples were withdrawn for analysis at 24 hour intervals. Results of analyses are given in Fig. 2.

Experiment XIII. Leaves of Kikuyu grass were used. Experiment commenced 8–11–43. The leaves were divided into four series. Two series served as duplicates in air and over these an air stream was passed for 96 hours; the remaining two series served as duplicate series in

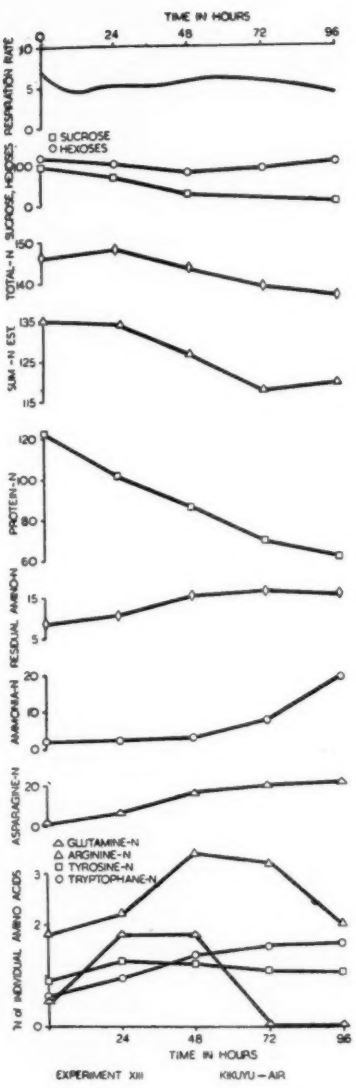

Fig. 3.

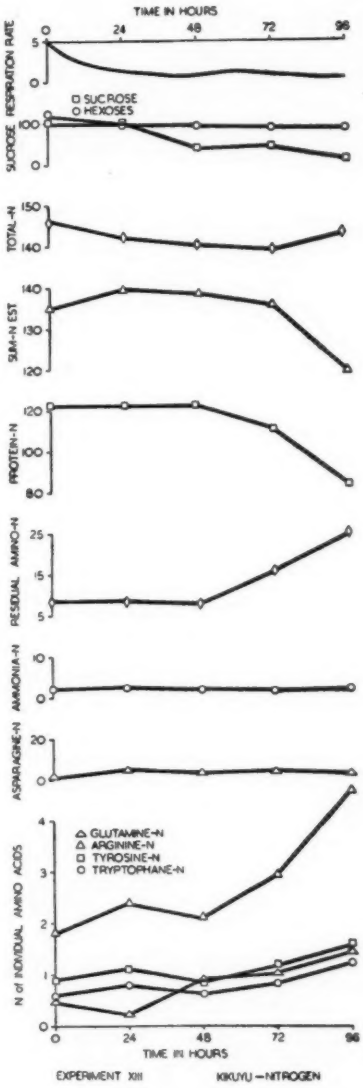

Fig. 4.

nitrogen and over these pure oxygen-free nitrogen was passed for 96 hours. The leaves in air were full yellow in colour on the 3rd day of starvation. Leaves in nitrogen were full green and turgid up to and including the 3rd day of starvation but on the 4th day were dry to touch. Analyses were performed in duplicate in each series. For all substances estimated the results for the two series in air agreed closely and mean values are reported. Similarly, the closely agreeing values of substances in the two series in nitrogen are reported as mean values. Results of analyses for the series in air are given in Fig. 3 and for the series in nitrogen in Fig. 4.

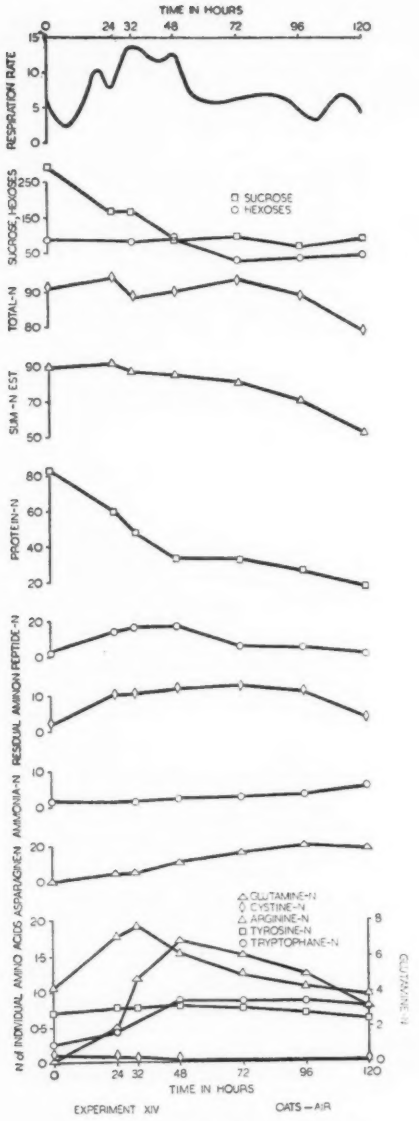

Fig. 5.

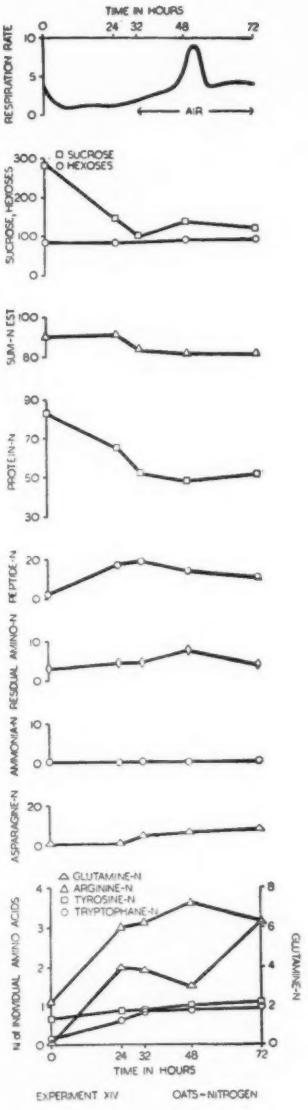

Fig. 6.

Experiment XIV. Leaves of a pure line of Algerian oats were used. Experiment commenced 26–7–43. The leaves were divided into two series. Over one series air was passed continuously for 144 hours. Leaves in air were full yellow on the 2nd day of starvation and had commenced to brown on the 3rd day. In this respect oat leaves differ from Kikuyu leaves; the latter do not become brown, but yellow, and if kept for long periods dry out still remaining

yellow. The second series of leaves were placed in a stream of pure nitrogen. The leaves were brown and showed signs of injury after 24 hours; after 32 hours in nitrogen the nitrogen stream was replaced by an air stream for a further 48 hours. The leaves remained brown, but still turgid during the sojourn in air. Results of analyses for the series in air are given in Fig. 5. and for the series in nitrogen in Fig. 6.

Analytical Methods.

In Experiments XI and XII all estimations were carried out on fresh material; in all other experiments estimations were made on leaf material dried rapidly in a current of air at 90° C. All estimations were performed in duplicate.

Respiration rate and amounts of *protein, residual amino-N, ammonia, glutamine* and *asparagine* were determined as described previously by Wood, Cruickshank and Kuchel (1943). *Total-N* was estimated by micro-Kjeldahl after digestion with H_2SO_4 containing salicylic acid and sod. thiosulphate. *Peptide-N* was estimated as described by Vickery *et al.* (1937). The abbreviation "Sum N-est" used in the figures is the sum of the nitrogen actually estimated in different compounds.

The individual amino acids were estimated in aliquots of protein-free filtrate after precipitation of the protein at pH 4·5 by trichloracetic acid and warming. 15 gm. fresh material or 1 gm. dried material were de-proteinized as above and the solution made up to a total volume of 25 ml. The aliquots used for analysis varied. Usually 0·5 ml. was used for arginine estimations, 3 ml. for tyrosine and tryptophane, and 15 ml. reduced by distillation to about 3 ml. for cystine estimations.

Cystine was estimated by Lugg's (1932) modification of the method of Folin-Marenzi, but using a total volume of 10 ml. in place of the 100 ml. recommended.

Arginine was estimated by Weber's (1930) modification of the Sakaguchi reaction according to the procedure recommended by Klein (1933). It was found that animal charcoal cleared the yellow-coloured solution without loss of arginine.

Tyrosine and tryptophane were estimated by Lugg's (1937) methods. Phenols and presumably extraneous indoles interfered especially with the tryptophane determinations and resort was made to extraction (1) with ether at pH 1 and (2) with toluene at pH 6·5. In oats the value for tryptophane after extraction with these solvents was about 70 p.c. of the unextracted value in all cases; in Kikuyu the extracted values were about 80 p.c. of the unextracted values in all cases. Charcoal could not be used to decolourize the extracts since it destroyed the amino acids in part; compensation for the colour was therefore necessary.

Aspartic acid. Attempts were made to estimate this compound in Experiment XI by means of the separation of copper and barium salts according to Brazier (1930) after hydrolysis of amides and distillation of ammonia from the protein-free filtrate. Amino-N in the fractions was determined with the Van Slyke apparatus. Trials with mixtures of the pure substances in amounts expected in the plant extracts showed that the method is not quantitative and losses of aspartic acid amounting up to 50 p.c. occurred; it was hoped that the values obtained would show the trends of these acids during starvation.

Respiration rate is reported as mg. CO_2 per 20 gm. original fresh weight per hour. All other substances are reported as mg. per 20 gm. original fresh weight.

DISCUSSION.

Comparison of Nitrogen Balance Sheets in Kikuyu and in Oats.

A nitrogen balance sheet is set out in Table 1 for Experiments XIII (Kikuyu) and XIV (oats). For each sampling time amounts of protein-N, peptide-N, ammonia-N, 2 × amide-N, residual α-amino-N, ¾ × arginine-N and ½ × tryptophane-N are given in addition to the total-N. Residual α-amino-N represents total α-amino-N *minus* the total amide-N and corrected for the anomalous behaviour of glutamine in the Van Slyke apparatus. ¾ × arginine-N represents guanidine-N not included in the figure for residual α-amino-N; similarly, ½ × tryptophane-N is a measure of the indole-N in tryptophane.

In Kikuyu (Experiment XIII) the sum of the nitrogen fractions listed in Table 1 accounts for approximately 90 p.c. of the total-N at each sampling period. No peptide-N could be detected in Kikuyu. Nitrogen not estimated includes nitrate-N, chlorophyll-N and nitrogen from lysine, histidine and prolins as well as from other sources. It is clear that these substances are present in relatively small amount. The sum of the N-compounds estimated shows a small decrease in amount throughout the course of the starvation period. This is evident also in other experiments with Kikuyu (*vide* Experiments XI and XII, Figs. 1 and 2 in the present paper and also Experiments IX and X described by Wood, Mercer and Pedlow, 1944). It would appear therefore that in Kikuyu grass transformations of nitrogen compounds are almost wholly accounted for by hydrolysis of proteins to amino acids and later oxidation of these, in part, to amides and ammonia.

Oats differs from Kikuyu grass in at least four respects. *First*, peptide-N, which cannot be detected in Kikuyu, forms a relatively large fraction of the total soluble-N; the amount of peptide-N rises to a maximum during early stages of starvation and later decreases. *Second*, the

sum of the N-fractions estimated, and also this sum expressed as a percentage of the total-N, decrease with time and especially during the later stages of starvation. It is apparent therefore that in this plant relatively large amounts of nitrogen compounds, not estimated here, play an important rôle in nitrogen metabolism during late stages of starvation. The nature of these compounds will be reported in a later paper. *Third*, protein degradation proceeds to a greater degree in oats than in Kikuyu, e.g. in the Experiments in Table 1, after 96 hours 50 p.c. of the original amount of protein in Kikuyu, but 69 p.c. of the original protein in oats has been broken down; after 120 hours 79 p.c. of the oats protein has disappeared. In all our experiments with Kikuyu grass (Wood, Cruickshank and Kuchel, 1943; Wood, Mercer and Pedlow, 1944) it has been found that not more than 55 p.c. of the original amount of protein is broken down even after a starvation period of 192 hours. Correlated with the more rapid breakdown of protein in oats than in Kikuyu is the observation that in oats the leaves were completely yellow after 48 hours starvation, whilst in Kikuyu, yellowing is not complete until after about 96 hours or later. It should also be remarked here that in oats the leaves become brown after full yellow stage has been reached, but leaves of Kikuyu do not become brown but remain yellow. *Fourth*, as will appear in the following section, secondary formation of glutamine apparently occurs in oats during starvation, but not in Kikuyu.

TABLE 1.

Starvation time (hours).	Protein-N.	Peptide-N.	Ammonia-N.	2 × (Glutamine plus Asparagine)-N.	Residual α-amino-N.	¾ × Arginine-N.	¼ × Trypto-phane-N.	Sum of foregoing fractions.	Total-N.
XIII Kikuyu									
0	122·3	nil	1·92	1·32	8·38	1·35	0·30	135·6	146
24	111·4	—	2·02	8·24	10·50	1·68	0·48	134·3	148
48	86·8	—	2·72	19·02	15·23	2·55	0·72	127·0	143
72	69·7	—	8·25	20·76	16·59	2·37	0·80	118·5	139
96	61·8	—	20·00	20·00	15·74	1·53	0·84	120·1	137
XIV Oats									
0	83·0	2·5	1·40	nil	2·84	0·81	0·08	90·6	91·0
24	59·8	14·6	1·17	5·80	10·20	1·32	0·33	93·2	93·6
32	47·3	17·9	1·68	8·98	11·01	1·41	0·37	88·7	88·2
48	34·4	17·8	2·16	16·64	13·58	1·17	0·68	86·4	89·9
72	33·9	5·7	2·74	21·18	15·61	0·90	0·58	80·6	93·4
96	25·6	4·1	3·03	25·64	11·88	0·81	0·59	71·7	89·0
120	17·8	1·5	5·02	21·36	7·12	0·72	0·53	54·1	78·5

It would appear, therefore, that some enzyme systems may differ both qualitatively and quantitatively in the two plants. In oats there occurs a more extensive breakdown of protein, including formation of peptides as well as of amino acids; and also some of the products, presumably amino acids, are transformed into N-compounds, not estimated here, and not present in large amount in Kikuyu. Apart from these differences the general course of metabolism during starvation appears to be the same in oats as in Kikuyu, i.e. amino acids decrease and amides and ammonia increase in amount, although much less ammonia is formed during late stages of starvation in oats than in Kikuyu.

Amounts of Amino Acids during Starvation.

During starvation in air there occurs with time a progressive decrease in amount of protein; this is accompanied first by an increase in amount of residual amino acids to a maximum value thereafter the amount of residual amino acids decreases. Throughout this period there occurs an increase in amount of asparagine and of ammonia. Since the latter compounds are not formed in nitrogen (see also Wood, Cruickshank and Kuchel, 1943; Wood, Mercer and Pedlow, 1944) it is apparent that their origin is from oxidation or transformation of amino acids and that they do not occur preformed in the protein molecule. Wood, Mercer and Pedlow (1944) have shown that during the period of formation of asparagine residual amino acid residues provide the main source of CO_2 evolved in respiration.

Estimations of amounts of individual free amino acids during starvation time in air (Figs. 1, 2, 3 and 5) indicate that the amount of each of these attains a maximum value and thereafter decreases. However, the time of attainment of maximum value is not the same for different amino acids. Furthermore, amounts of some amino acids decrease more rapidly than others. The relative order of utilization of the acids estimated is the same in all experiments and the series in order of utilization is cystine, glutamine, arginine, tyrosine, tryptophane.

TABLE 2.

Table showing differences in amounts actually found between sampling periods of various amino acids, together with amounts of the same acids expected from protein hydrolysis. All amounts in mg. nitrogen per 20 gm. original flesh weight. *Plus* signs refer to increases, *minus* signs to decreases in amount during the inter-harvest intervals. Data from experiments in air only.

Inter-sampling period (hrs.).	Protein plus peptide.	Aspartic acid. Calc.	Aspartic acid. Found.	Glutamic acid. Calc.	Glutamic acid. Found.	Arginine. Calc.	Arginine. Found.	Tyrosine. Calc.	Tyrosine. Found.	Tryptophane. Calc.	Tryptophane. Found.
Experiment XI.											
0–24	15·33	0·75	+2·68	1·23	+0·52	2·34	+0·35	0·27	+0·20	0·35	+0·23
24–48	16·42	0·80	+1·95	1·31	nil	2·55	+0·08	0·30	+0·23	0·37	+0·30
48–72	12·27	0·60	nil	0·98	nil	1·90	−0·28	0·22	−0·02	0·27	+0·18
72–96	7·17	0·36	+4·00	0·57	nil	1·09	−0·18	0·14	−0·20	0·17	−0·16
96–144	3·10	0·15	+7·16	0·25	nil	0·48	−0·12	0·05	−0·17	0·07	−0·04
Experiment XII.											
0–24	7·97	0·39	+1·93	0·64	−0·46	1·20	+0·82	0·18	+0·20	0·14	+0·14
24–48	7·18	0·35	+0·76	0·57	nil	1·10	+0·58	0·17	+0·17	0·13	+0·19
48–72	9·15	0·45	+3·30	0·73	nil	1·38	−1·16	0·21	−0·02	0·17	+0·17
72–96	7·90	0·39	+3·69	0·63	nil	1·20	−0·54	0·18	−0·04	0·14	−0·16
96–120	2·26	0·11	−4·14	0·18	nil	0·34	−0·36	0·05	−0·16	0·04	+0·04
120–168	3·53	0·17	−3·67	0·28	nil	0·54	−0·18	0·08	−0·23	0·06	−0·14
Experiment XIII.											
0–24	10·9	0·53	+2·78	0·89	+0·68	1·69	+0·36	0·25	+0·44	0·20	+0·30
24–48	24·6	1·20	+5·38	1·97	+0·01	3·81	+1·16	0·56	−0·08	0·44	+0·48
48–72	17·1	0·84	+1·78	1·37	nil	2·65	−0·24	0·41	−0·09	0·31	+0·16
72–96	7·9	0·39	−0·38	0·63	nil	1·22	−1·12	0·18	−0·07	0·14	+0·08
Experiment XIV.											
0–24	11·1	0·54	+1·90	0·89	+1·00	1·73	+0·68	0·26	+0·10	0·20	+0·22
24–32	9·2	0·45	+0·25	0·74	+1·34	1·40	+0·14	0·21	+0·01	0·41	+0·39
32–48	13·0	0·64	+2·76	1·04	+1·07	2·02	−0·34	0·30	+0·03		
48–72	12·6	0·62	+2·66	1·01	−0·39	1·95	−0·32	0·29	−0·03	0·23	−0·06
72–96	9·9	0·49	+2·71	0·79	−0·48	1·51	−0·14	0·23	−0·06	0·18	nil
96–120	10·4	0·51	−1·17	0·83	−0·97	1·63	−0·14	0·24	−0·09	0·19	−0·12

The degree to which different amino acids are utilized during starvation becomes clearer from examination of the data in Table 2. In this Table we have set out the decrease in amount of protein between each sampling period in each experiment in air. Using the known amino acid analyses of grass proteins (Chibnall, 1939), we have calculated the increase in each amino acid which would be expected between each sampling period provided that it was not removed in any way. This value, shown as "calculated" value for each amino acid in Table 2, is compared with the differences actually found between each sampling period for each amino acid; the latter are labelled "found" in the Table.

In calculation of the amino acid values the following values, each expressed as percentage of the total protein-N, were used; aspartic acid-N, 4·9 p.c.; glutamic acid-N, 8·0 p.c.; arginine-N, 15·5 p.c.; tyrosine-N, 2·3 p.c.; tryptophane-N, 1·8 p.c. The following conclusions may be drawn:

Asparagine. In Experiment XI, where aspartic acid was estimated approximately, it has been shown that the aspartic acid content closely parallels that of asparagine (Fig. 1). The "found" values for aspartic acid-N in Table 2 have been derived by taking the asparagine amide-N as a measure of amount of aspartic acid. From Table 2 it is clear that the increases

in amounts of aspartic acid actually formed between each sampling period are much greater than the amounts expected from protein hydrolysis. This finding substantiates our observations that asparagine is not produced in leaves during starvation in nitrogen as well as the long established view that formation of the amide is secondary in origin, and that at least the amide-N is derived from oxidation of amino acids.

Glutamine. In Kikuyu utilization of glutamine during starvation is very rapid; in all experiments with this grass glutamine cannot be detected after the second day of starvation by the methods of analysis used. Examination of Table 2 reveals that in all instances the amount of glutamine present is markedly less than that expected from protein hydrolysis. It is probable, therefore, that in this grass the glutamine present during starvation arises from hydrolysis of protein and is not of secondary origin. This supposition is borne out by the data of Experiment XIII for the leaves kept continuously in nitrogen (Fig. 6) and in which there is a progressive increase in amount of glutamine, while the amount of asparagine remains approximately constant.

In oats, on the other hand, glutamine is present during the whole of the starvation period of 120 hours; and Table 2 reveals that during early stages of starvation glutamine is present in amounts greater than can be accounted for from hydrolysis of proteins. Secondary origin of the amide in part is therefore probable. In this respect oats are more nearly akin to barley, for it is evident from Yemm's (1937) data for leaves of the latter, that glutamine is present in amounts larger than can be accounted for by protein hydrolysis. Kikuyu on the other hand more closely resembles tobacco, investigated by Vickery *et al.* (1937); during starvation of these leaves much asparagine but little glutamine is formed. In rhubarb, Vickery *et al.* (1939) have found that glutamine only, but not asparagine, is formed during starvation. It is probable, as suggested by Wood, Mercer and Pedlow (1944), that the nature and amount of enzyme systems concerned in amide formation vary in leaves of different species.

The behaviour during starvation of the other amino acids estimated is the same in all experiments and in both oats and Kikuyu.

Cystine is present in small amount in all cases and could be detected only by analysis of fresh leaves; no cystine could be found in the dried leaves of Experiment XIII. Cystine is present, in amounts capable of estimation, only during the first one or two days of starvation; in all cases the maximum value occurred at the beginning of the starvation period. In all cases the amount of cystine present is much less than that expected from the amount of protein hydrolysed. Wood and Barrien (1939) found that during starvation of rye grass leaves attached to the plant, protein-S decreased in amount and an equivalent amount of sulphate-S accumulated in the leaves. During the process of protein synthesis in the light, Petrie and Wood (1938b) found that cystine did not increase as the amount of other amino acids increased. It appears, therefore, that cystine is more readily oxidized than other amino acids.

Arginine. Previous determinations of amounts of arginine in plants have been made chiefly in conifer seedlings. Suzuki (1900) considered that arginine was produced synthetically at the expense of other amino acids, as well as being derived from protein hydrolysis. He considered arginine to play much the same rôle as does asparagine. He also stated that transformation of arginine was greater and took place more quickly than that of asparagine. Schulze (1896) previously had considered that arginine might be formed synthetically as well as from protein breakdown but realized that formation of new proteins in the seedlings might affect the position, i.e. the new-formed proteins might be poorer in basic-N than seed proteins. In lupin seedlings Schulze found that the amount of arginine in the sap never exceeded that which must have been produced by protein hydrolysis. Mothes (1929) could find no evidence of secondary formation of arginine in conifer seedlings. His analyses confirmed Schulze's opinion that the newly formed protein of the seedling was relatively poorer in basic-N than the protein of the seeds. Mothes also stated that arginine was subjected to deamination only with difficulty in the presence of mono amino acids. Klein and Taubóck (1932) found little utilization of arginine in pine seedlings, but that some of the arginine was used in metabolic reactions in leguminous seedlings.

In all our experiments the course of arginine metabolism during starvation is uniform. The maximum arginine content is attained on about the second day of starvation and thereafter the amount of arginine decreases rapidly. Reference to Table 2 shows that on each sampling occasion the amount of arginine present is less than that expected from protein hydrolysis; the relative "calculated" and "found" figures suggest rapid utilization of arginine, presumably resulting in formation of asparagine.

Tyrosine. Tyrosine is utilized during starvation much less readily than is arginine, cystine or glutamine. The maximum tyrosine content is attained later than the arginine maximum and occurs between the second and third days of starvation. Table 2 shows that during the first two days of starvation the amount of tyrosine actually found is only slightly less than that expected from protein hydrolysis. Later tyrosine is utilized to a greater extent.

Tryptophane. Tryptophane is utilized less readily during starvation than is tyrosine. The maximum tryptophane content occurs between the third and fourth days of starvation (Figs. 1, 2, 3 and 5) and Table 2 shows that up to this time the tryptophane actually found is equal to or only slightly less than that expected from protein hydrolysis. Thereafter, like tyrosine, tryptophane disappears more rapidly than it is formed during protein hydrolysis.

Relations between Proteins and Amino Acids.

The picture which emerges from the foregoing discussion on leaves subjected to starvation is similar to that obtained by Petrie and Wood (1939a and b) for plants grown in the light and subjected to varying treatments of ammonium salts and of water content. There it was found that the relations between proteins and amino acids could be expressed by a curve concave to the amino acid concentration axis; asparagine and glutamine showed high positive correlation with residual amino acids (Wood, 1942), but they found that one amino acid, viz., cystine, did not increase as residual amino acids increased in amount; and they considered that at least one factor contributing to the relations found between proteins and amino acids was the preferential oxidation of this amino acid as the concentration increased, thus bringing about a disproportionality among the amino acids which were now no longer present in equivalent proportions, so that a given amount of protein was in equilibrium with a greater residual amino acid content.

Our findings in the present paper of varying degrees of utilization of amino acids during starvation support this view and do not suggest apolar adsorption of the kind advocated by Phillis and Mason (1942).

We may say, therefore, that all the data we have obtained in our series of experiments in air during both protein synthesis and protein degradation are explicable on the view that there exists a steady state relationship between protein and amino acids based on the law of mass action, but complicated by the preferential oxidation of some amino acids at a rate more rapid than that of others. The latter property apparently depends on whether the rate of synthesis of amino acids is greater or less than rate of oxidation since Wood, Cruickshank and Kuchel (1943) found that when the carbohydrate content of leaves was very high then there is little or no change in amounts of protein and amino acids during the first few days of starvation. The mechanism outlined here implies a reversible relation between proteins and amino acids, the same enzyme catalyzing both forward and back reactions.

However, the relatively simple mechanism outlined in the last paragraph, whilst apparently true for conditions in air, is not sufficient to account for the data when changes in the leaves in an atmosphere of nitrogen are taken into account. The outstanding feature of leaves starved in an atmosphere of nitrogen is the fact that whilst loss of carbohydrates proceeds as rapidly in nitrogen as in air, yet the change in amounts of protein and of amino acids is very small in nitrogen whilst it is large in air (see also Wood, Cruickshank and Kuchel, 1943; and Wood, Mercer and Pedlow, 1944). It would appear also that the amount of protein hydrolysis in nitrogen is bound up in large measure with degree of injury to the leaves. In the fourth paper of this series (Wood, Mercer and Pedlow), where hydrolysis in nitrogen was somewhat more extensive than in other of our experiments, we had noticed it to be associated with drying of the cut ends of the leaves. This feature is marked in Experiment XIII of the present paper. The leaves were fresh and turgid till the end of 48 hours of starvation in nitrogen, and during this period no detectable hydrolysis of protein occurred. Drying of the cut ends commenced on the third day, and was extensive on the fourth day of starvation; this was correlated with increased protein hydrolysis. Similarly in oats, where browning and injury to leaves are apparent after only a short sojourn in nitrogen, there is a relatively rapid protein hydrolysis. In nitrogen, amino acids only accumulate and there is no formation of asparagine.

It is apparent from our experiments that the protein level is not dependent on respiration rate as suggested by Richards (1938). In leaves in nitrogen, CO_2-production falls to a very low level indeed without perceptible change in contents of either protein or amino acid during starvation. During starvation in air there

is no correlation between respiration rate and protein content. Also Wood and Petrie (Petrie and Wood, 1938a and b; Wood, 1942), found that during synthesis of protein in leaves of plants supplied with ammonium salts in the light there was no evidence that the protein content was determined by respiration rate.

In all experiments carried out by the firstnamed author of this paper and his co-workers respiration rate exhibited high positive linear correlations with amino acid content. In the case of leaves of plants in which synthesis of protein was taking place the leaves all possessed high amino acid concentration following application of ammonium salts; in the case of starving leaves it was shown by Wood, Mercer and Pedlow (1944) that the bulk of the CO_2 produced during the climacteric period was derived from amino acids and their transformations. Vickery and Pucher (1939) have studied the distribution of carbon in rhubarb leaves cultured under various conditions, and show that respiration draws upon components other than carbohydrates and that these are probably amino acids.

In the above cases no correlation between proteins and respiration rate could be found. It remains, therefore, to bring into some sort of coherent picture the observations of different authors who have described high linear correlations between protein content and respiration rate (Gregory and Sen, 1937; Richards, 1938; Petrie and Williams, 1938; Wood, 1941), and to account for which Richards suggested a reciprocal relation between proteins and amino acids whereby a definite rate of CO_2-evolution was necessary to maintain a definite level of protein. Gregory and Sen used plants subjected to varying degrees of nitrogen and potassium deficiency. They found that in nitrogen deficiency respiration rate was correlated with amino acid and protein contents, the two latter variables themselves being correlated; in potassium deficiency respiration rate was correlated with protein and with carbohydrate contents. In the nitrogen-deficient plants carbohydrate content was high, but in potassium-deficient plants was low. Richards, using the same technique, investigated the effects of phosphorus deficiency: he found that in normally manured plants and in mild cases of phosphorus deficiency respiration rate was correlated with protein and amino acid contents, the two latter themselves being positively correlated; in extreme phosphorus deficiency respiration rate was correlated with protein content and carbohydrate content. In the former case carbohydrate content was high and in the latter case relatively low.

It is noteworthy that the carbohydrate content was high in cases where correlations exist between respiration rate and amino content. Barker (1936) has shown in potatoes that respiration rate is not correlated with carbohydrate in a linear manner, but the relationship can be expressed by a hyperbolic function as expected from the Michaelis equation. Also Wood (1942), increased the carbohydrate content of grass leaves by adding sucrose solutions to pots in which relatively N–deficient plants, already high in carbohydrate content, were growing. Under these circumstances amino acid and protein contents remained practically unchanged, but carbohydrate content reached high values. Respiration rate under these conditions remained approximately constant and independent of carbohydrate content. Lack of correlation between respiration rate and carbohydrate content in some of the data of Gregory and Sen and of Richards does not imply therefore lack of real effect, but simply that the effect may be approaching its maximal limit. When carbohydrate content is high, the relation between respiration rate and amino acids becomes more marked; only when carbohydrate content is low, as in potassium and phosphorus deficiency, is there a marked correlation between respiration rate and carbohydrate content.

It has been shown above that all the CO_2 produced in respiration does not arise from carbohydrates during protein synthesis, in deficiency of essential elements and during starvation, but that part is derived from amino acid residues. It is difficult in most cases to disentangle the proportions of CO_2 derived from

the different sources. *Whilst therefore the view that a given rate of CO_2-evolution can only maintain a definite quantity of protein cannot be substantiated, in all cases, the data of all observers are in harmony with the hypothesis that protein content of leaves in air may be determined by the rate at which carbohydrates are passing through an oxidative cycle.* Under these circumstances protein content might be expected to be correlated only with that portion of the respiratory CO_2 which is derived from carbohydrates. As pointed out above, respiration rate is not correlated with carbohydrate content when the latter is high, but only when carbohydrates are relatively low. The above hypothesis is supported by all the starvation experiments of the firstnamed author and his co-workers *loc. cit.;* in these the trend of protein content with time closely follows that of sucrose, except when sucrose content is very high when no loss of protein occurs.

The mechanism whereby such a relation between protein content and oxidation of carbohydrates is maintained is speculative. It has been pointed out earlier that proteins and amino acids exhibit relations which can be interpreted in terms of Mass Law, but modified by the fact that some amino acids are oxidized more readily than others (Wood, 1942). It would appear a reasonable assumption that a definite level of protein can be maintained *in air* provided that synthesis of the most readily oxidized amino acids takes place at the same rate as their oxidation. Such a state of affairs would obviously depend, in the last analysis, on the rate of breakdown of carbohydrates which provide the intermediary substrates in amino acid formation, provided sufficient nitrogenous initial substances were also present.

Although the hypothesis outlined in the last two paragraphs accounts satisfactorily for relations between proteins, amino acids, respiration rate and carbohydrates in air, it is not sufficient to account for their behaviour when leaves are starved in an atmosphere of nitrogen. In nitrogen protein content is quite unrelated to carbohydrates which decrease in amount as rapidly in nitrogen as in air; in nitrogen protein content is also unrelated to respiration rate, and as shown in this and previous papers, the protein content decreases and amino content increases only very slightly, except in cases of injury.

It is apparent, therefore, that in nitrogen proteolytic enzymes in some way are prevented from coming into contact with their protein substrates, whilst in air this occurs with greater facility. It is known that many native proteins are not broken down by enzymes until they have been denatured and Linderström-Lang (1939) has suggested that this has its origin in steric hindrances, especially in blockage or combination of peptide groups in the molecule in various degrees, and that in protein hydrolysis an initial reaction, enzymatic or otherwise, must take place prior to fission of the peptide bonds. It would appear, however, that "steric hindrance" in a wider sense than this might retard protein hydrolysis such as is found in leaves starved in nitrogen. This appears clearest in the case of chloroplast protein. Wood, Cruickshank and Kuchel (1943) and Wood, Mercer and Pedlow (1944), have shown that the contents of chloroplast protein, chlorophyll and ascorbic acids remain approximately constant over long periods in leaves starved in nitrogen. On starvation in air, on the other hand, chloroplast protein, chlorophyll and ascorbic acid all decrease in amount at approximately the same rate; but if the carbohydrate content is very high, then breakdown of all these compounds in air is retarded. Recent work (Weier, 1938), has focussed attention on the existence of a pattern within the chloroplast in which protein, chlorophyll, carotinoids, ascorbic acid and phosphatides are all interlocked components. Strain (1938) has also pointed out that xanthophylls and chlorophyll are not readily oxidized in living leaves, but undergo rapid oxidation on injury to the leaves or when the chloroplast structure

is destroyed. Similar patterns, but without the pigments, probably occur in the cytoplasm. We suggest that it is this pattern which provides the steric hindrance which protects the protein from hydrolysis by enzymes in an atmosphere of nitrogen.

In nitrogen we can picture this pattern as static, not subject to oxidation of the components and the protein protected from enzymes by the existence of the pattern. In air, on the other hand, all the components of the pattern are subject to oxidation, and, as pointed out in the last paragraph, decrease at approximately the same rate during starvation. The static pattern present in nitrogen is now replaced by a dynamic one in which each individual component undergoes both synthesis and degradation; on the temporary breaking of the pattern during oxidation we may picture proteolytic enzymes coming into contact with their substrates and causing the relations between proteins and amino acids we have found to exist in leaves. Whether the components of the pattern remain present in any given amount will depend upon whether their rates of synthesis are equal to that of their oxidation, and this in turn will be determined in large measure by the rate of carbohydrate breakdown. As carbohydrate content falls the original pattern becomes fragmented and amongst other things protein becomes more liable to hydrolysis; this is the picture we put forward to account for the "decrease in organization resistance" or "increase in hydrolysis facility" first suggested by F. F. Blackman to explain the climacteric rise in respiration in leaves.

SUMMARY.

Amounts of various free amino acids, as well as of other nitrogenous compounds, have been determined in leaves of Kikuyu grass (*Pennisetum clandestinum*, Hockst) and Algerian oats (*Avena sterilis*, L) starved in atmospheres of air and of nitrogen. Respiration rate and carbohydrate contents were also determined.

Examination of nitrogen balance sheets indicates that in Kikuyu leaves starved in air transformations of nitrogen compounds are accounted for by hydrolysis of proteins to amino acids and subsequent oxidation of these to asparagine and ammonia. Whilst these processes also occur in oats, the latter differs from Kikuyu in the formation of undetermined nitrogenous compounds during late stages of starvation; in accumulation of peptides; more extensive breakdown of protein; and in secondary formation of glutamine.

During starvation in air, amino acids are preferentially oxidized, the order of utilization of those determined being cystine, glutamic acid, arginine, tyrosine and tryptophane.

During starvation in nitrogen very little breakdown of protein and accumulation of amino acids occurs unless the leaves are injured. Amino acids only accumulate and no asparagine or ammonia is formed.

The data of other papers by the authors, and those of other workers are investigated in the light of the present findings and the following conclusions are drawn as to the nature of relations between proteins and amino acids in leaves:

(a) the presence of a pattern in which proteins, pigments, phosphatides and ascorbic acid are interlocked components, provides steric hindrance which prevents protein hydrolysis in nitrogen. In air all components of the pattern are subject to oxidation to a degree depending upon the rate of carbohydrate oxidation; the dynamic character of the pattern in air allows enzymes to come into contact with their protein substrate.

(b) the relations between protein and total amino acid concentration can be expressed by a curve concave to the amino acid concentration axis; at least part of the concavity results from the observed facts that some amino acids are oxidized more rapidly than others. A reversible relation between protein and amino acids is implied, modified by preferential oxidation of some amino acids.

(c) the curve relating protein and amino acid content is not directly affected by respiration rate; but observations of various workers under widely different experimental conditions are all in harmony with the view that protein content is determined by the rate at which carbohydrates are utilized in an oxidation cycle. It is suggested that maintenance of a definite protein level depends upon whether syntheses of the most readily oxidized amino acids from carbohydrates and nitrogenous substrates take place at a rate at least equal to their rates of oxidation.

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THE ACTION OF 3, 3′-ETHYLIDENE-BIS (4-HYDROXY COUMARIN) AS A BLOOD ANTI-COAGULANT

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The occasional necessity in surgery to administer a blood anti-coagulant has led to the search for suitable compounds. Most of the proposed substances have a narrow margin between efficacy and toxicity and, therefore, until recently only heparin, one of the physiological regulators of blood coagulation, found wide clinical application. The unwieldy method of administration and the cost, however, are limiting factors in its usefulness. Much attention has, therefore, been paid to 3, 3′-methylene-bis (4-hydroxy coumarin)-dicumarol which was isolated by Campbell and Link (1941) during their work on the haemorrhagic principle present in spoilt sweet clover hay. Clinical reports show that, when dicumarol is introduced in the circulation, it delays blood coagulation by causing hypoprothrombinaemia. In contrast to heparin, it has no effect on shed blood. Apart from the risks of using compounds increasing the blood coagulation time, dicumarol has the disadvantage of prolonged action which can be relieved only temporarily by blood transfusion. Recently, it was reported (Fantl, 1943) that 3, 3′-ethylidene-bis (4-hydroxy coumarin), referred to later in this paper as E.D.C., was also effective in producing hypoprothrombinaemia and, since it was observed that it had a lower toxicity than dicumarol, its application as an anti-coagulant was suggested.

PREPARATION OF E.D.C.

4-hydroxy coumarin was prepared by fusion of sodium with acetylsalicylic methyl ester according to Pauly and Lockemann (1915). The high yield recorded by these workers, however, could not be realized and it was observed that the large amount of sodium was unnecessary. For 100 gm. of ester only 7 gm. of sodium were used. The average yield was 18 p.c. of the theoretical. With the older technique Stahmann *et al* (1941) obtained approximately the same yield. The condensation was carried out with paraldehyde. 2·45 gm. 4-hydroxy coumarin were dissolved in 350 ml. boiling water, 4 ml. each of paraldehyde and 10 N sulphuric acid were added and the mixture kept boiling for 5 minutes. After 24 hours, standing at room temperature, the precipitate was collected and washed with water until free of acid. The crude material was dissolved in approximately 5 ml. of warm benzene. After addition of an equal volume of methylated spirits, the compound crystallized out. A further recrystallization yielded 2·1 gm. of colourless crystals having a melting point of 169° C. Anschütz (1909) found 165° C. The compound is soluble in water to the extent of approximately 0·015 p.c. at 20° C. In aqueous suspension or aqueous pyridine solution the substance consumes one equivalent of alkali per mol. (350 gm.). The biological tests were carried out on normally fed rabbits having body weights between 1,500 to 2,000 gm. E.D.C. was administered intraperitoneally in freshly prepared solution having a concentration of 0·8 p.c. E.D.C. and sodium carbonate. A few minutes after preparation the sodium salt begins to crystallize. The time between consecutive injections was 24 hours. The blood samples were taken either from the ear vein, but more frequently from the jugular vein. The prothrombin estimations were carried out by the technique of Quick (1938) on whole plasma and plasma diluted with saline to 12·5 p.c. All samples of plasma contained N/50 sodium oxalate. Equal volumes of thromboplastin emulsion and M/40 calcium chloride solution were mixed prior to use and 0·2 ml. of the mixture was added to 0·1 ml. plasma at 37·5° C.

That the delayed clotting of the plasma was due to the reduction in prothrombin content could be shown by addition of normal plasma to the deficient specimens.

The results obtained in the last two experiments indicate that the delayed clotting was due only to lack of prothrombin and not to other interfering substances. In the latter case the clotting time of the mixtures would be greater than 21 seconds.

TABLE 1.

Influence of intraperitoneal injection of 3, 3'-ethylidene-bis (4-hydroxy coumarin) on coagulation of rabbit's plasma.

Dosage in mg per kg. body weight.	Hours after administration. 0	4	24	48	72
	Coagulation time in seconds.				
(I) 17 + (II) 19	20	29	78†	143	19
(I) 24	19	28	58	18	18
(I) 26 + (II) 16	22	26	120†	1,200	32
(I) 27	21	—	51	35	—
(I) 34 + (II) 32	19	—	88†	480	19
(I) 40	17	—	31	18	—
(I) 41	19	—	76	22	18
(I) 41 + (II) 19	22	17	71†	600	46
(I) 44	22	23	130	45	17
(I) 53	16	—	59	15	—
(II) 24	17	—	48	—	—
(III) 103	18	—	82*	—	17
(III) 108	22	—	∞*	—	20
(III) 109	24	—	240*	—	21

The coagulation time is given for 12·5 p.c. diluted plasma, except the results marked with an asterisk which refer to whole plasma. The daggers indicate that the animal received a second injection after the blood sample was taken.

The Roman numerals indicate the number of injections given; the Arabic figures the amount of substance in mg. per kg. body weight.

TABLE 2.

	Clotting times.	
Normal rabbit's plasma A	9 seconds	21 seconds (with 12·5 p.c. plasma)
Pathological plasma I	> 4 hours	
" II	240 seconds	
87·5% " I + 12·5% A	19 seconds	
87·5% II + 12·5% A	15 seconds	

From the results recorded in Table 1, it is apparent that there are rather wide variations in the ability of E.D.C. to produce a hypoprothrombinaemia. Further, it will be noted that, in the majority of the animals, even 4 hours after the injection an influence was observed. The maximal effect was seen 24 hours later with a return to normal on the following day. Fatal haemorrhages were observed only in two animals; one received 80 mg. per kg. E.D.C. in two injections, the other 140 mg. per kg. in three injections. Other animals, receiving similar doses recovered, in spite of a loss of about 95 p.c. prothrombin.

As the results tabulated below indicate, E.D.C. is much less potent than dicumarol.

TABLE 3.

Influence of intraperitoneal injection of dicumarol on coagulation of rabbit's plasma.

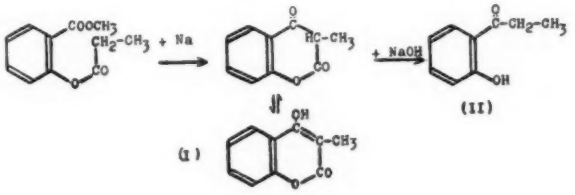
Dosage in mg. per kg. body weight.	Hours after administration. 0	4	24	48	72
	Coagulation time of 12·5% of plasma in seconds.				
0·5	20	—	33	27	—
1·5	22	29	69	53	22
1·8	22	—	79	112	92
2·8	26	—	—	53	—
4·3	40	—	—	240	—

E.D.C. was administered orally to rabbits by Jansen and Jensen (1942) and Lehmann (1943). The former found it inferior to dicumarol, whilst Lehmann obtained equal effects with both compounds.

In view of a certain similarity in constitution between the alkylidene dicoumarins and 2-methyl 1,4-naphthoquinone, a compound with vitamin K activity, it might be possible that dicoumarins antagonize vitamin K action. It was therefore of interest to test 3-methyl 4-hydroxy coumarin, a substance which shows still closer structural relationship to the quinone.

Preparation of 3-methyl 4-hydroxy coumarin (I)[1].

20 gm. propionyl salicylic methyl ester were heated in an oil-bath at 170° C. Small portions of sodium totalling 1·4 gm. were successively introduced, vigorous stirring being maintained until all the sodium was dissolved. The reaction product, a semi-solid mass, was cooled to room temperature, dissolved in water and separated from the oily layer. The latter was shaken with water until neutral. The aqueous solution was acidified with hydrochloric acid and the precipitate collected and washed with water until free of mineral acid. A yield of 4–4·5 gm. was obtained.

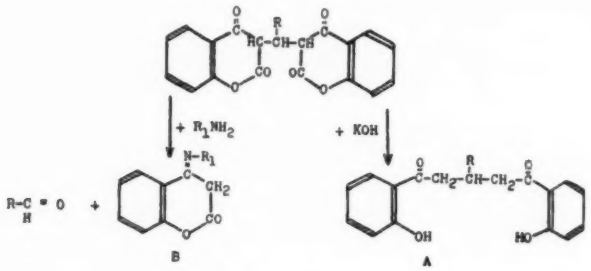


The substance was boiled with small volumes of absolute alcohol to remove salicylic acid. It was recrystallized from absolute alcohol and after treatment with charcoal a pure product with the following properties was obtained: M.P. 228° C. (in evacuated capillary); ferric chloride reaction in alcohol, light brown; slightly soluble in water; molecular weight in camphor, 194—calculated 176; equivalent weight by titration with standard alkali in water or aqueous pyridine 177 and 181; for analysis dried in vacuum over phosphorus pentoxide at 100° C., C 67·8 H 4·5—calculated C 68·16 H 4·58. Although the molecular weight estimation gave too high a value, the constitution was established by degradation with alkali in the following manner: *o*-propionyl phenol (II) 2 gm. of 3-methyl 4 hydroxy coumarin were mixed with 25 ml. of 10 p.c. sodium hydroxide. The sodium salt precipitated but, on heating, went into solution. The mixture was boiled under reflux for 12 hours, was then neutralized and distilled in steam; the volatile oil was taken up in ether, dried over sodium sulphate and the ether removed. The residue represented the keto-phenol in an amount of 1·4 gm. In aqueous suspension it gave a purple ferric chloride reaction. The ketone was dissolved in absolute alcohol and a concentrated aqueous solution of 0·7 gm. hydroxylamine hydrochloride was added. The reagents were refluxed for 3 hours, while potassium carbonate was gradually added in order to maintain neutrality towards methyl red. Water was poured into the mixture and the oxime recrystallized from methanol. M.P. 95° C.; ferric chloride produced an ink colour; nitrogen content 8·22 p.c. (micro-kjeldahl) calculated 8·48 p.c.

3-methyl 4-hydroxy coumarin was dissolved in the required amount of sodium hydroxide and injected intraperitoneally into rabbits. Amounts up to 200 mg. per kg. body weight in a single injection were without influence on the coagulation time of rabbit's plasma 24 and 48 hours after administration. Amounts up to 350 mg. per kg. in three injections were likewise without effect. Doses between 490 mg. and 550 mg. per kg. body weight produced a severe hypoprothrombinaemia, but the animals died between 2–3 days after the injection. These reactions, following the high doses cannot be considered as a specific effect of the compound. Thus, in tolerated doses—3-methyl 4-hydroxy coumarin can be considered as inactive. Jansen and Jensen (1942) and Lehmann (1943) have tested this compound after oral administration. The former found it to be inactive, whereas the latter worker observed a slight activity. This negative result offers no proof for the assumption of competition between the alkylidene dicoumarins and vitamin K for the same substrate. Another reaction mechanism was considered. It is known that dicoumarins can undergo two different routes of degradation by basic reagents.

[1] This compound has apparently been prepared by Jansen and Jensen (1942). The m.p. is given as 218–220° C.

Strong alkali produces a keto-phenol A, whilst primary amines react with the formation of B. In the latter case an aldehyde will be liberated. It seems possible that reaction leading to B takes place in the liver and that the aliphatic aldehyde formed might be responsible for the observed biological reaction. Some evidence can be offered for this assumption from *in vitro* experiments.



Formaldehyde was prepared from polyoxymethylene by dry distillation at 180° C. in a nitrogen current and was absorbed into cold water. Acetaldehyde was made from paraldehyde by addition of 10 p.c. 10 N. sulphuric acid and distilled into cold water. To 0·3 ml. oxalated plasma, the aldehydes in equimolecular concentration of $0{\cdot}32–1{\cdot}5 \times 10^{-2}$ millimol. were added and the volume made up to 1 ml. The final solution contained 0·8 p.c. sodium chloride and N/50 sodium oxalate. The samples were kept for an hour at room temperature and afterwards the plasma coagulation time was measured in the usual manner.

TABLE 4.

	Without addition		Addition of			
	30 p.c. plasma.	12·5 p.c. plasma.	Formaldehyde. 0·4 mg.	Formaldehyde. 0·13 mg.	Acetaldehyde. 0·6 mg.	Acetaldehyde. 0·2 mg.
Human plasma (3 specs.)	21–23	45	35–41	25–28	38–45	25–26
Rabbit plasma (9 specs.)	10–13	17–22	17–33	13–17	17–18	13–14

The figures represent coagulation time in seconds.

DISCUSSION.

Link *et al.* (1943) have suggested that the prothrombin lowering effect of dicumarol was due to salicylic acid, which is a breakdown product. The conclusion was derived from experiments with rats kept on vitamin K deficient diet, and it has been reported (Shapiro *et al.*, 1943) that salicylates in adequate dosage induce prothrombinopenia in man. It seems doubtful, however, whether the amount of salicylic acid which can be formed by the breakdown of effective doses of dicumarol will influence the prothrombin level of normally fed animals. In fact, a comparison between the effective amounts of dicumarol and E.D.C. shows that in the rabbit much larger amounts of the latter are necessary to produce the same effect as dicumarol. Since the molecular weights of these compounds differ only by 4 p.c., and it can be assumed that their breakdown in the body will follow similar lines, it seems very unlikely that salicylic acid is the main toxic agent. From the results of my experiments it is suggested that dicoumarins act by liberation of an aliphatic aldehyde in the liver. It appears probable that the inhibition by aliphatic aldehyde was at least partly due to prothrombin reduction. From available information, it seems that formaldehyde, in small concentration, does not influence the conversion of fibrinogen to fibrin (Chargaff, 1943). An influence, however, on thromboplastin cannot be ruled out since Fisher (1936) assumed that formaldehyde decreases the clotting effect of organ extracts.

SUMMARY.

Intraperitoneal injection of 3, 3′-ethylidene-bis (4-hydroxy coumarin) into normally fed rabbits produces hypoprothrombinaemia to a lesser degree than equimolecular amounts of the corresponding methylene compound. Recovery following the administration of the ethylidene derivative, however, is more rapid.

3-methyl 4-hydroxy coumarin is without effect on blood coagulation in tolerated doses.

Formaldehyde and acetaldehyde increase the coagulation time of plasma. That these aldehydes might be responsible for the biological effects of the alkylidene dicoumarins, rather than salicylic acid, is suggested.

Acknowledgments. Appreciation is expressed to Dr. E. Singer for the taking of blood samples, and to Mrs. D. Buckley, B.Sc., Department of Organic Chemistry, University of Sydney, for the combustion analysis.

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THE ANTI-INSULIN ACTION OF ANTERIOR PITUITARY EXTRACTS

by J. F. NELSON

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(Accepted for publication, 26th January, 1944.)

Houssay (1937) was the first to demonstrate that animals injected with anterior pituitary extracts develop an insensitivity to the hypoglycaemic action of insulin. Later, Young (1937) suggested the name "glycotropic" factor for the active principle and showed that it is distinct from the diabetogenic principle and unrelated to prolactin, gonotropic and thyrotropic factors.

From studies in rabbits in which the liver was excluded from the circulation, Himsworth (1938) proved that the pituitary glycotropic factor inhibited the action of insulin in accelerating the removal of blood glucose by the peripheral tissues.

Marks (1936) compared the action of insulin in promoting muscle glycogen deposition in normal and pituitary treated animals. The spinal eviscerated cat, receiving a continuous infusion of glucose, was used and it was found that insulin was less effective in animals previously injected with anterior pituitary extract. This suggested a direct antagonism against the peripheral action of insulin.

Recently, Gemmill and Hamman (1941) have reported on the effect of insulin on glycogen deposition and glucose utilization by isolated muscles. These observations were made on the rat's diaphragm suspended in Ringer's solution in Warburg flasks. This technique suggested a means for obtaining precise information as to the site of the inhibition of insulin action by anterior pituitary extracts. Thus comparisons could be made on the effect of insulin on glycogen deposition in isolated muscle from normal and pituitary treated animals.

METHODS.

The rats were killed by a blow on the head. The diaphragm was immediately excised and immersed in cooled Ringer's solution (approx. 10° C.). It was then divided by cutting along the central ligament. The posterior portion was discarded.

The two anterior sections were washed in fresh Ringer's solution, dried quickly and placed in Warburg vessels containing 3 ml. of Ringer's solution; one section was used as a control and to the other insulin was added.

The Ringer's solution was similar to that of Gey and Gey (1936) with a pH of 7·5–7·6. It contained 0·2 p.c. glucose but magnesium salts were absent. In most of the experiments sodium bicarbonate was also omitted. Here the vessel contents were equilibrated by passing oxygen for 2–3 minutes. In the experiments using a solution containing bicarbonate, it was saturated with 5 p.c. carbon dioxide and 95 p.c. oxygen before use, and the manometers were filled with this gas. The temperature of the bath was maintained at 37·5° C.

In all experiments the vessels were shaken at the rate of 80 times per minute. The muscles were incubated for 3 hours, then removed from the vessels, dried quickly on filter paper and placed in 30 p.c. potassium hydroxide. Glycogen was determined by the method of Good, Kramer and Somogyi (1933) and recorded as glucose.

For the insulin experiments 1 ml. (60 units) of a solution of insulin (C.S.L.) was added to 89 ml. of Ringer's solution so that the tissue was in contact with two units of insulin. The original sample of insulin contained 0·1 p.c. trieresol added as preservative.

All animals were fed up to the time of experiment. Those treated with pituitary extract were injected intraperitoneally 24 hours beforehand with 3 ml. of a crude saline extract, equivalent to 1·5 gm. of fresh gland.

Since oxygen consumption could readily be determined throughout the incubation, these values are also recorded as a matter of interest. The pH of the Ringer's solution before and after incubation was determined by means of the glass electrode.

RESULTS.

The results of glycogen determination and oxygen consumption are given in Table 1.

TABLE 1.

Normal.						A.P.E. treated.					
Glycogen p.c.			Q_{O_2}			Glycogen p.c.			Q_{O_2}		
No insulin.	Insulin.	Difference.	No insulin.	Insulin.	Difference.	No insulin.	Insulin.	Difference.	No insulin.	Insulin.	Difference.
0·18	0·43	0·25	1·04	1·06	0·02	0·30	0·34	0·04	1·01	0·95	0·06
0·40	0·79	0·39	1·03	1·03	0·00	0·12	0·08	0·04	1·05	1·07	0·02
0·21	0·49	0·28	0·95	0·98	0·03	0·16	0·14	0·02	0·96	0·97	0·01
0·15	0·51	0·36	1·16	1·19	0·03	0·10	0·06	0·04	1·04	1·09	0·05
0·29	0·52	0·23	0·97	1·08	0·11	0·14	0·09	0·05	1·05	1·00	0·05
0·34	0·50	0·16	1·00	1·02	0·02	0·08	0·14	0·06	1·03	1·00	0·03
0·33	0·50	0·17	1·00	1·02	0·02	0·16	0·16	0·00	0·99	1·01	0·02
0·15	0·43	0·28	0·98	1·04	0·06	0·14	0·24	0·10	1·10	1·07	0·03
0·20	0·46	0·26	1·02	1·01	0·01	0·14	0·28	0·14	1·01	1·04	0·03
0·29	0·53	0·24	0·98	1·00	0·02	0·12	0·11	0·01	1·05	1·01	0·04
0·20	0·40	0·20	1·03	1·00	0·03	0·17	0·14	0·03	1·08	1·04	0·04
0·23	0·44	0·21	0·96	1·04	0·08	0·13	0·13	0·00	1·06	1·05	0·01
0·12	0·36	0·24	1·00	1·01	0·01	0·19	0·20	0·01	1·06	1·03	0·03
0·22	0·45	0·23	1·08	1·05	0·03	0·19	0·23	0·04	1·07	1·00	0·07
0·25	0·56	0·31	1·05	1·04	0·01	0·10	0·15	0·05	1·08	1·07	0·01
						0·15	0·12	0·03	0·97	0·97	0·00
		Mean 0·254			Mean 0·032			Mean 0·041			Mean 0·031

From these results it appears:

1. The administration of anterior pituitary extract inhibits the action of insulin in promoting the deposition of glycogen in the isolated muscle.
2. Oxygen consumption is not affected.

Further, in animals treated with anterior pituitary extracts the initial muscle glycogen values are lower than normal.

In the above experiments the Ringer's solution did not contain bicarbonate. In a few cases the pH fell to as low as 6·6 over the 3 hours period and no appreciable glycogen deposition in the presence of insulin was noted.

This can be readily explained since Fantl, Rome and Nelson (1942) have shown that the optimum pH for glycogen breakdown is $6·6 \pm 0·1$ while, at above pH 7·2, this breakdown is very much retarded.

In a further small series of experiments a Ringer's solution containing 2·27 gm. sodium bicarbonate per litre was used. Here the pH at the end of 3 hours had not changed appreciably from its initial value of 7·6, and invariably glycogen deposition in the presence of insulin occurred in normal tissues. However, as shown in Table 2, pituitary treated animals still failed to respond.

TABLE 2.

Glycogen p.c.

Normal.		A.P.E. treated.	
Without insulin.	With insulin.	Without insulin.	With insulin.
0·28	0·58	0·11	0·17
0·20	0·59	0·20	0·27
0·28	0·70	0·13	0·17
0·27	0·68	0·12	0·14
0·27	0·70		

SUMMARY.

The anti-insulin effect of anterior pituitary extracts has been investigated on the isolated diaphragm muscle of rats.

The action of insulin in promoting glycogen storage is greatly impaired in muscles isolated from animals previously injected with anterior pituitary extract.

Oxygen consumption is unaffected by either insulin or anterior pituitary extract.

The effect of pH on glycogen deposition *in vitro* is discussed.

Acknowledgment. I should like to express my sincere thanks to Dr. A. B. Corkill for suggesting these investigations and for his help and guidance throughout the course of the work.

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GLYCOLYSIS IN EXTRACTS OF OYSTER MUSCLE

by GEORGE FREDERICK HUMPHREY

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(Accepted for publication 24th March, 1944.)

In 1929 Kobayashi showed that when minced oyster muscle was incubated at 37° C., lactic acid was formed. However, no attempt was made to determine what factors were necessary for acid production. The present study was undertaken to discover more about the glycolytic processes which can occur in oyster muscle, both in continuation of the programme already outlined (Kesteven and Humphrey, 1941), and because of the lack of information about glycolysis in the invertebrates.

MATERIALS.

The oysters used were 2–3 year old stock of *Saxostrea commercialis* from the C.S.I.R. Experimental Farm at Georges River, N.S.W.

The adenylic acid (AA) and rabbit glycogen were from B.D.H. London. The oyster glycogen was prepared by digesting oyster meat with 60 p.c. KOH followed by several precipitations from final concentrations of 50 p.c. alcohol and 80 p.c. acetic acid. The white product was dried *in vacuo* over $CaCl_2$ and NaOH and contained no nitrogen or phosphorus.

The diphosphopyridine nucleotide (D.P.N.) was prepared by the method of Williamson and Green (1940).

The starch was A.R. (soluble) from B.D.H.

The p-hydroxy diphenyl, lithium lactate and pyruvic acid were all prepared in the laboratory.

METHODS.

The enzyme solutions were prepared by dissecting the adductor muscles of a few oysters, grinding with one and a half times their weight of ice-water and a little sand; after standing 5 mins. the mixture was centrifuged and the supernatant fluid used at once.

Solutions were then added to Warburg manometer cups to give the following final concentrations:

A.A. (as a solution in phosphate buffer of pH 7·3)	0·35 mg./ml.
Mg++ (as $MgCl_2$)	0·20 "
D.P.N.	0·20 "
Oyster glycogen	10·00 "
KH_2PO_4–Na_2HPO_4 buffer of pH 7·3	0·05 M
$NaHCO_3$	0·025 M

Water to give a final volume of 3·0 ml.

Finally 0·5 ml. of the enzyme solution was added to the side-bulb and the manometers filled with 5 p.c. CO_2–95 p.c. N_2 gas mixture. After equilibrium and tipping the manometers were shaken for two and a half hours at 37° C.[1] At the end of that time the contents of each flask were treated with 1·5 ml. of ice-cold 24 p.c. trichloracetic acid and after standing 5 mins. in the refrigerator the precipitates were centrifuged off and the supernatant fluids analysed for pyruvic and lactic acids by the methods of Friedmann and Haugen (1943), and Barker and Summerson (1941) respectively.

RESULTS.

From Table 1 it can be seen that in the absence of either D.P.N. or glycogen there was no lactic acid produced. The manometric figures for lactic acid were calculated from the gas evolution, uncorrected for CO_2 retention or changes due to phosphate esterification. The former was neglected because it is usually small and the latter could not be calculated with any certainty, since it is not known which esters are elaborated under the experimental conditions. Although

[1] The pH of the reaction mixture was tested as suggested by Dixon (1943, p. 60). Before use the buffers were checked electrometrically with the double hydrogen electrode.

it was expected that the amount of lactic acid found chemically would be less than that calculated from the manometric readings, the discrepancy is rather large. Also when A.A. was not added more gas was evolved than when it was added; in this and other experiments (not quoted), the amount of acid found chemically was always approximately the same whether A.A. was added or not. Probably the extract already contained enough; however A.A. was always added to give the best chance for maximal glycolysis. Since the discrepancy between the chemical and manometric figures was so large it was thought that there might be an accumulation of some other acid; pyruvic seemed to be the most likely, and when tested for was found to be present.

TABLE 1.

Extent of glycolysis under different conditions.

	μg. lactic acid.	
	Chem.	Manomet.
Complete experiment	119	372
Without enzyme	0	72
„ substrate	0	176
„ A.A.	107	600
„ D.P.N.	0	128
„ A.A. or D.P.N.	0	244

TABLE 2.

Accumulation of pyruvic and lactic acids.

	μg. Pyruvic.	μg. Lactic.	μg. Total acid.*	
	Chem.	Chem.	Chem.	Manomet.
Complete experiment	32	172	204	280
Without enzyme	0	0	0	80
„ substrate	0	0	0	245
„ A.A.	91	96	187	784
„ D.P.N.	0	0	0	140
„ A.A. or D.P.N.	0	0	0	220

* Since pyruvic and lactic acids have approximately the same molecular weights, it is possible to calculate the figure for total acid (assuming pyruvic and lactic the only acids formed), from the μl. of CO_2 simply by multiplying by 4, and this holds no matter what is the ratio between the two acids.

Even here the discrepancies seem too large to be explained by the change due to phosphate esterification. The remainder of the experiments reported in this paper were carried out to discover what factors influenced the production of these two acids.

TABLE 3.

Effect of time on acid accumulation.

		μg. Pyruvic.	μg. Lactic.	μg. Total acid.	
		Chem.	Chem.	Chem.	Manomet.
Complete experiment	2 hrs.	149	90	239	540
Without A.A.	„	204	81	285	604
Complete experiment	4 hrs.	371	136	507	672
Without A.A.	„	446	90	536	1,024
„ enzyme	„	0	0	0	112
„ substrate	„	0	0	0	276

From Table 3 it can be seen that even after 4 hours pyruvic acid was still present, and in contrast to the previous experiment the quantities were larger than the corresponding ones of lactic acid. This is probably because different enzyme solutions were used in the two experiments; it was not possible, however, to keep enzyme solutions overnight, even in the refrigerator. Extracts of acetone powders were also inactive.

The results in Table 4 show that when either borate (Clark, 1927), or NaH_2PO_4–Na_2HPO_4 buffer of equivalent pH was substituted for KH_2PO_4–Na_2HPO_4 buffer no acid production took place, i.e. both phosphate and potassium are needed. Also in the absence of Mg. (which could be replaced by an equi-molar concentration of Mn) no acid was produced. When this was repeated it was found that sometimes the amount of acid produced was less in the presence of Mn and sometimes greater.

TABLE 4.

Effect of inorganic substances.

	μg. Pyruvic. Chem.	μg. Lactic. Chem.
Complete experiment	112	67
Without Mg.	0	0
Mn. substituted for Mg.	223	156
NaH_2PO_4 substituted for KH_2PO_4	0	0
Borate substituted for phosphate	0	0

The effect of changing the substrate is shown in Table 5. Oyster glycogen could be replaced only by starch or rabbit glycogen, the latter proving to be the most effective of the three.

TABLE 5.

Effect of different substrates.

	μg. Pyruvic. Chem.	μg. Lactic. Chem.
Oyster glycogen	328	218
Rabbit „	451	323
Starch	143	81
Fructose	0	0
Galactose	0	0
Glucose	0	0
Mannose	0	0

Fluoride or iodoacetate at a concentration of M/30 completely inhibited acid production (Table 6); M/1,000 iodoacetate behaved similarly but M/1,000 NaF was insufficient for complete inhibition. The slight production of acid in the latter instance may have been due to the removal of the fluoride by Mg. In all cases the inhibitors were added directly to the manometer cups and were not placed in the side-bulb with the enzyme before tipping.

TABLE 6.

Effect of inhibitors.

	μg. Pyruvic. Chem.	μg. Lactic. Chem.
Complete experiment	352	192
M/30 NaF	0	0
M/1,000 „	45	142
M/30 Iodoacetate	0	0
M/1,000 „	0	0

In Fig. 1 are shown the shapes of the reaction curves under various conditions. Curves such as these were obtained a number of times, the sharp delayed rise in gas production being characteristic; this "induction" phenomenon has been noted by Needham *et al.* (1937) under other conditions.

DISCUSSION.

Although in many respects the results presented for oyster muscle differ from those obtained previously on other animals, there are certain features in common, *viz.*, the dependence of acid production on the presence of Mg (Mn), K and inorganic phosphate and also the lack of acid production from hexoses in the absence of added hexokinase.

The inhibitions by NaF and iodoacetate can both be explained by assuming the usual glycolytic cycle. However, the simultaneous accumulation of both pyruvate and lactate cannot be explained in the same way. Even if the lactate is produced from pyruvate by any of the known reactions (Stern, 1940), it is possible that the equilibria in this animal are different from those found in ani-

mals such as rats, rabbits, etc. In the absence of information about the metabolism of pyruvate by oyster muscle there is no basis for speculation about the phenomena observed.

However, there is still the question of the large disparity between the chemical and manometric figures for total acid production. In view of the high concentrations of buffers used and also the magnitude of the amount of acid found chemically, it is not likely that the pH changed very much (Dixon, 1943, p. 85). Meyerhof (1926), reports in some instances, quite large discrepancies between chemical and manometric figures for lactic acid production, even after the latter figures have been corrected for phosphate esterification changes. A partial explanation of the discrepancies obtained with oyster muscle may be the occurrence of the following anaerobic dismutation (Krebs and Johnson, 1931). $2\ CH_3\ CO\ COOH + H_2O = CH_3\ CHOH\ COOH + CH_3\ COOH + CO_2$. Owing to the difficulty of determining acetic acid it will be necessary to conduct larger scale experiments to test this hypothesis.

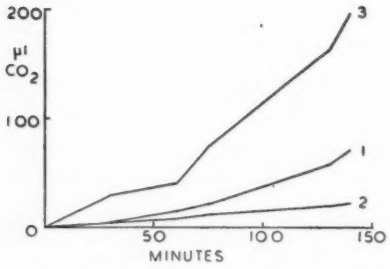

Fig. 1. Showing the course of CO_2 evolution under different conditions: 1. Complete experiment. 2. Without enzyme. 3. Without adenylic acid.

Although there was never a very great difference in the amounts of total acid, estimated chemically, when A.A. was added and when it was not, there was, however, a marked difference in the CO_2-evolutions (Fig. 1). This may possibly be due, in part, to differences in phosphorylative reactions which occur during the change: glycogen to lactic and pyruvic acids; this explanation is purely tentative, however, since no information is yet available as to just what phosphorylated intermediates are formed.

SUMMARY.

The production and accumulation of pyruvic and lactic acids have been demonstrated in ice-water extracts of oyster muscle.

More gas is evolved from the bicarbonate buffer in which the reaction is carried out than can be explained by the amount of these two acids found by chemical means.

The following have been shown to be essential parts of the system: phosphate, K, Mg or Mn and D.P.N.

Oyster glycogen, rabbit glycogen and starch, but not fructose, glucose, galactose, or mannose can act as substrate for the reaction.

Fluoride and iodoacetate inhibit acid production.

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THE MODE OF ACTION OF QUININE ALKALOIDS AND OTHER ANTIMALARIALS

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The primary object of this work is to establish an hypothesis for the mode of action of quinine in malaria.

It is well known that *in vitro* quinine has a toxic action on enzymes and unicellular organisms. *In vivo* it possesses similar powers but its action is generally considered to be indirect. Apart from its action on plasmodia it can influence the cells of the host. However, very little is known about the mechanism in either case. With a view to obtaining some information on these processes a detailed study of the pharmacology of quinine and related compounds was undertaken.

A. SMOOTH MUSCLE.

Experimental.

Rat's intestine has been found to be most satisfactory because the spontaneous movements were not too gross and the reactions to calcium and quinine were more regular. The method of Magus was employed. The bath contained 15 ml. To investigate the interaction between quinine and other drugs a standard effect of the latter was obtained and the Tyrode in the bath was then changed several times. Quinine, etc., was now added and allowed to act for 6–8 minutes, the original drug was then added in the presence of quinine and the response noted. The bath was now washed out several times (referred to as "w.o." in Figs.) and the action of the original substance again tested at intervals. Quinine was employed as the bisulphate, quinidine and cinchonine as the hydrochloride. Atebrin was supplied by the kindness of Dr. Albert.

Results.

In an earlier paper (Keogh and Shaw, 1943) it has been shown that quinine causes a relaxation of the longitudinal muscle and a slight increase of rhythmical movement. Similar results have been obtained with quinidine, cinchonine, plasmoquin and atebrine (see Figs. 1 and 2). Later it was found that small doses, 0·25 mg. in the bath, produced a rise in tonus.

Fig. 1. Rat's intestine. Ca = 3 mg. $CaCl_2$. Q = 1 mg. Quinine hydrochloride. W.O. = Wash out with Tyrode. Time = 30 sec.

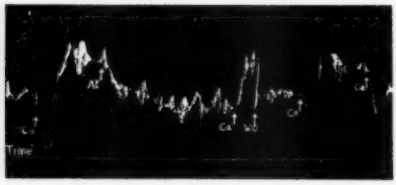

Fig. 2. Rat's intestine. Ca = 3·5 mg. $CaCl_2$. At. = 1 mg. Atebrine. W.O. = Wash out with Tyrode. Time = 30 sec.

Since the publication of that paper it has been observed that whilst calcium normally causes a relaxation of smooth muscle, after the treatment of the intestine with quinine the addition of calcium produces a contraction of the longitudinal muscle. This is referred to in future as the "calcium reversal". This reversal has been obtained with all the substances enumerated above (see Figs. 1 and 2). A possible reason for this calcium reversal was presented when it was

[1] This work was carried out by the aid of funds supplied by the University of Melbourne and the National Health and Medical Research Council of Australia.

found accidentally that if a piece of intestine were in Ca-free Tyrode the relaxation subsequent to the addition of Ca to the bath became less and less and was finally replaced by a contraction (Fig. 3). On substituting normal Tyrode the addition of Ca gave the usual relaxation.

The difference in the time interval of the manifestation of the calcium reversal between quinine and Ca-free Tyrode should be noted. Treatment of the gut with quinine for about 3–5 minutes is sufficient, whilst to remove the calcium of the gut by washing requires about 30 minutes. This would suggest that one reason why alkaloids are so reactive is that they possess the power of quickly reacting with the cell surface (*vide* fragility experiments) or of penetration into the cell interior.

Fig. 3. Rat's intestine. Ca = 3 mg. $CaCl_2$. N. Tyrode = Normal Tyrode. Time = 30 sec.

After the addition of each Ca the bath was washed out with Ca-free Tyrode until this was replaced by normal Tyrode.

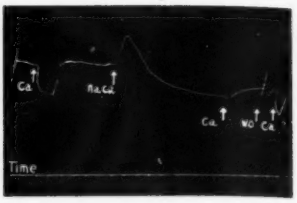

Fig. 4. Rat's intestine. Ca = 5 mg. $CaCl_2$. Na Cit. = Sodium citrate 15 p.c. W.O. = Wash out with Tyrode. Time = 30 sec.

To test further the hypothesis that the calcium reversal is due to the removal of calcium ions, sodium citrate, sodium oxalate or sodium tartrate was added to the bath in excess (usually 50 p.c.) of the amount necessary to "neutralize" the calcium ions present in the bath. In each case the reversal was obtained (see Fig. 4). If excess oxalate is not added the reversal is not obtained but the subsequent relaxation is usually less than the control. The optimum doses of quinine, plasmoquin, cinchonine and atebrine to give the reversal were found to be 1–2 mg., that of quinidine 6 mg. If less were used the reversal was not obtained but only a diminution of the amount of relaxation. This is evident from Fig 5 where the dose of quinine has gradually been increased. An excess of quinine, etc., was usually toxic, i.e. excessive relaxation and cessation of all movement resulted. The antagonism of quinine and calcium was shown by the fact that if a mixture of 2 mg. of quinine and 2 mg. of calcium were added to the bath the calcium reversal was not obtained. The reversal is independent of the anion used and has been obtained with the bisulphate, hydrochloride and citrate.

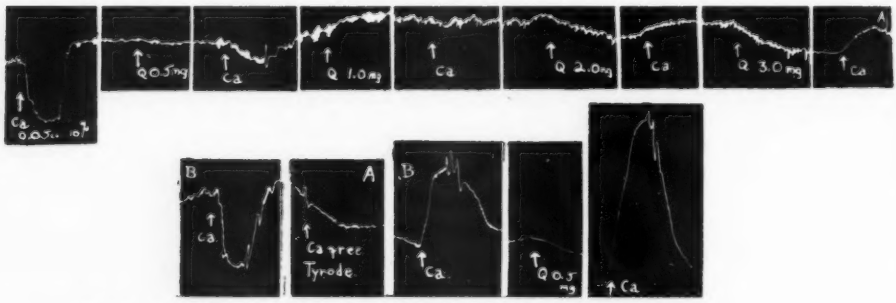

Fig. 5. Rat's intestine. Ca = 5 mg. $CaCl_2$. Q = Quinine bisulphate.

Between each panel is an interval of 5 mins., except between AB when there is an interval of 20 mins.

The quantitative aspect of this calcium-quinine antagonism is also shown by the following experiment. Mixtures of quinine and calcium in varying amounts were prepared and different dosages were added to the bath. Three to five minutes later a dose of calcium which previously had given a relaxation of the intestine, was now added. The results of this experiment, which

are recorded in Table 1, show that if excess calcium is present the "calcium reversal" is not obtained and as the proportion of calcium is decreased or the quinine increased in the mixture a stage is reached when the addition of calcium to the bath produces at first neither a contraction nor relaxation of the muscle. Then as the proportion of quinine increases (or calcium decreases) the "calcium reversal" is obtained. This occurs when the proportion of quinine bisulphate to calcium chloride in the mixture is 1: 1·5 on a weight basis.

The relaxation on the addition of quinine commences when about 1–2 mg. of quinine is added to the bath. The calcium reversal also commences when this amount of quinine has been added to the bath. As the bath contains about 2·5 mg. of calcium, this agrees fairly well with the ratio given in the Table. The intestine bathed in Ca-free Tyrode is more sensitive in its response to quinine; half the dose of quinine produces relaxation and "calcium reversal" and the size of the calcium contraction is much greater (Fig. 5).

TABLE 1.

Experiment No.	Composition of mixture added to bath. mg. Q.	mg. Ca.	Ratio Q: Ca.	Response of intestine to calcium. (—— = No response).
I	0·2	7·0	1: 35	Fall
II	0·25	5	1: 20	Fall
III	0·6	8	1: 13	——
	0·9	12	1: 13	——
	1·2	16	1: 13	——
IV	0·75	5	1: 7	Fall
	1·5	10	1: 7	——
	2·0	14	1: 7	——
V	0·4	2	1: 5	Fall
	0·8	4	1: 5	Fall
	1·0	5	1: 5·	——
	1·5	7	1: 5	——
VI	0·45	1	1: 2	Fall
	0·90	2	1: 2	Rise
	1·85	3	1: 2	Rise
VII	0·9	2	1: 2	Fall
	1·8	4	1: 2	Fall
	2·7	6	1: 2	Fall
VIII	0·9	1·5	1: 1·5	Fall
	1·9	3·0	1: 1·5	Slight rise
	2·85	4·5	1: 1·5	Rise
IX	0·9	1	1: 1	Fall
	1·9	2	1: 1	——
	2·85	3	1: 1	Rise
X	0·95	0·7	1: 0·8	Fall
	1·9	1·4	1: 0·8	Rise
XI	0·25			Fall
	0·5			Fall
	0·75			——
	1·0			Rise
	1·5			Rise
	2·5			Rise
	3·5			Toxic

To test the specificity of the calcium reversal the following were tested in varying concentrations: sparteine, morphine, nicotine, histamine, luminal, methylene blue, *p*-phenylene diamine, sulphanilamide, strychnine, brucine, and alstonia. Of these strychnine and brucine gave a reversal; with methylene blue and alstonia a reversal was only obtained with difficulty but the relaxation was abolished, i.e. the intestine did not react to added calcium. The other substances did not influence the extent of calcium relaxation.

The reversal is not brought about with other metals, i.e. the effects of potassium and barium are reduced but not reversed in the presence of quinine. An attempt was made to see whether potassium-free Tyrode reversed the effect of a second addition of potassium. The experiment was not successful because the individual variation in response to potassium was too great; an addition of potassium to a normal intestine sometimes produced a contraction and sometimes a relaxation.

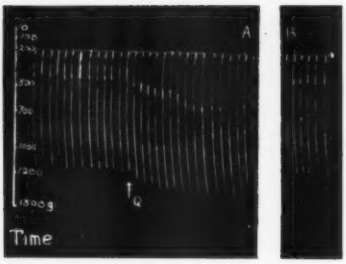

Fig. 6. Tibialis anticus, Dog. Dial and ether. Q = 7 mg./kg. quinine bisulphate. Time = 30 sec. Time A–B = 2 hrs.

Tension in gm. contraction downwards.

B. STRIATED MUSCLE.

(a) A preparation similar to that described by Brown (1938) was used. The dog's tibialis anticus muscle was attached by a thread from its tendon to an isometric lever. The sciatic nerve was divided and the peroneal nerve stimulated every 10 seconds by means of an induction coil. The injections were made intra-arterially, with an intact circulation.

Results.

In agreement with the results of Harvey (1939) quinine (7 mg./kg.) caused an increase of about 30 p.c. in the tension developed by the tibialis anticus when stimulated by the peroneal nerve (Fig. 6), or when the muscle was stimulated directly in a curarized preparation.

Similar results have been obtained with quinidine, cinchonine and atebrine. In the case of quinidine the maximum increase in tension takes a long time to develop. The rise with atebrine is not so marked and excessive doses tend to be toxic.

To continue the investigation of the hypothesis derived from a consideration of the results obtained with smooth muscle, namely that quinine alters the nature of the calcium ions within the cell, the action of citrate, etc., was investigated on this preparation. At first some difficulty was experienced in obtaining results analogous to those of quinine.

Sodium citrate $2H_2O$ 9 p.c. was used in these experiments. 2–3 c.c. of this solution produced at first a brief contracture of the muscle followed by an increase in the tension developed on stimulation of the peroneal nerve. Smaller doses given during this latter phase further increased the tension (Fig. 7). This contracture is apparently due to an effect on the nerve or nerve ending as it is not obtained in a curarized preparation which otherwise gave a response identical with that with quinine. It is interesting to note that the contracture brought about by citrate is similar to that produced when acetyl choline is injected. This is evident if Figs. 7 and 8 are compared (see Harvey, 1939). A similar result has been obtained with sodium tartrate (15 p.c.).

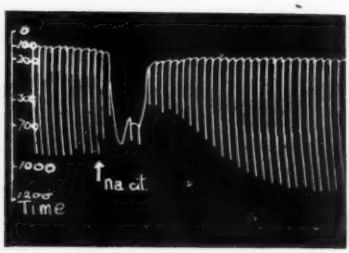

Fig. 7. Tibialis anticus, Dog. Dial and ether. Na Cit. = 2 c.c. sodium citrate 9 p.c. Tension in gm. Time = 30 sec.

The quinine effect with sodium oxalate (2·5 p.c.) could not be obtained; instead a progressive decrease in tension was obtained. If, however, the preparation was curarized the quinine effect was obtained. The failure to obtain a positive result on nerve stimulation was attributed to the fact that the oxalate precipitated too great a proportion of the calcium at the neuromuscular junction and so interfered with the transmission of the impulse. (Calcium is necessary for transmission at the neuro-muscular junction: Eccles, 1942).

The other quinine alkaloids and atebrine gave responses on the curarized preparation identical with those obtained with quinine.

Harvey (1939) has also shown that eserine produces a large increase in the tension. This increase is abolished if quinine is administered. This we have confirmed (Fig. 9) and extended the observation to include cinchonine and atebrine (Fig. 9).

With quinidine, citrate and tartrate we have only been able to obtain slight temporary reductions of the eserine-enhanced tension.

Many unsuccessful attempts were made to prevent a quinine effect of previous administration of calcium. This will be considered in the discussion.

It is of interest to note that strychnine, which produced the calcium reversal with smooth muscle, also gave a quinine effect on striated muscle.

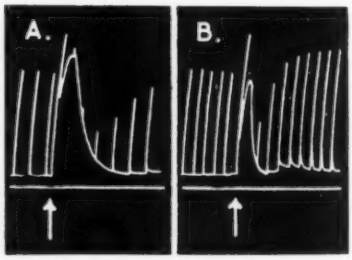

Fig. 8. Cat decerebrate = 2·8 kg. A = 0·5 mg. A.Ch. B = 0·5 mg. quinine. One minute later 0·5 mg. A.Ch. (at arrow) contraction upwards.

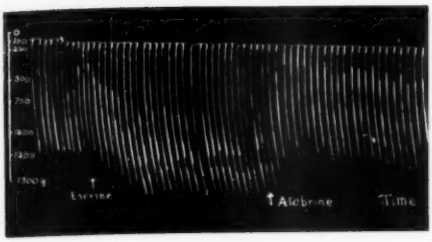

Fig. 9. Tibialis anticus, Dog. Dial and ether. Eserine = 0·02 mg./kg. eserine. At. = 2 mg./kg. atebrine. Time = 30 sec. Tension in gm.

It should be stressed that adequate controls for pH and osmotic pressure were carried out. Solutions of pH 3–10 were without effect, likewise an injection of 5 p.c. NaCl. The tension developed by the muscle on stimulating the peroneal nerve remained constant for periods up to an hour. Occlusion of the popliteal artery for 20 minutes produced only a 5 p.c. drop in the tension developed.

(b) *Sartorius muscle.* Harvey (1939) has shown that quinine decreases the excitability of the isolated sartorius muscle to directly applied induction shocks. This we have confirmed and in addition we have shown that if the calcium content of the fluid in the bath is trebled this effect is not produced. That is to say, calcium exhibits antagonism towards quinine. It was found necessary to add the calcium to the muscle bath about 15–20 minutes before the quinine. Presumably this period was necessary to allow the calcium to reach the site of action.

C. CIRCULATION.

Intact Animal.

Experimental. Cats were quietened with a minimum amount of nembutal. The dose was administered intraperitoneally and the amount varied for each animal according to its needs. The anaesthesia was completed with ether. This produced a preparation with a high blood pressure and suitable for the exhibition of vaso motor reversal (Hutchinson *et al.*, 1939). Blood pressure was recorded by means of a cannula in the carotid artery and drugs were injected intravenously. In all cases the optimum doses of adrenaline and calcium chloride (5–8 p.c.) were determined, the drug under investigation was then administered and after a suitable time interval the doses of adrenaline and calcium chloride were repeated.

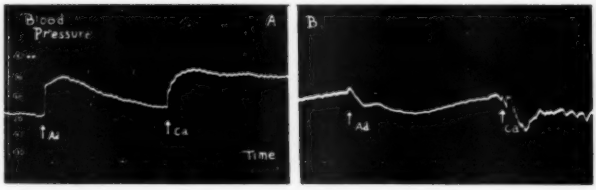

Fig. 10. Blood pressure, Cat, 2·5 kg. nembutal and ether. Ad. = Adrenaline 1 c.c. 1: 10^5. Ca = Calcium chloride 1 c.c. 8 p.c. Between A and B 60 mg. quinine bisulphate in 2 divided doses. Time interval: 7 min. Time = 30 sec.

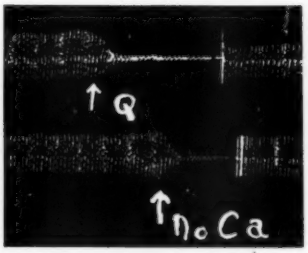

Fig. 11. Frog's heart, Straub's preparation. Q = Quinine 1: 2 × 10^5. No Ca = Calcium-free Ringer.

Results. It has been shown previously that after the administration of quinine adrenaline, which normally produces a rise in blood pressure, yields a fall (Keogh and Shaw, 1943). It is less well known that the intravenous injection of calcium chloride produces a rise in blood pressure. We have shown that this rise is reversed after the administration of quinine (Fig. 10). In the case of quinidine and cinchonine this reversal of the actions of adrenaline and calcium is more difficult to produce; it is more usual to obtain only an abolition of the rise. Here again we have been able to show the similarity in the properties of quinine and its related alkaloids and their interaction with calcium.

Frog's Heart Straub Preparation.

From Fig. 11 it will be seen that the action of quinine and calcium-free Ringer is similar. Several unsuccessful attempts were made to antagonize the quinine by increasing the calcium content of the Ringer solution.

D. MISCELLANEOUS.

In this section there are set out several experiments which may have some bearing on the action of quinine in the body.

(a) *Effect on Fragility of the Corpuscles.*

It has been suggested that quinine weakens the red cell membrane. Ponder and Abels (1936) showed that the haemolysis of red blood cells by saponins was facilitated by quinine.

Experimental. The method employed to determine the fragility was to prepare a series of tubes containing 2·5 ml. of saline ranging from 0·52 p.c. to 0·32 p.c. in steps of 0·02 p.c. To these tubes 0·1 ml. of blood was added. One hour later tubes were centrifuged and the degree of haemolysis noted.

To determine the effect of quinine on haemolysis *in vitro* quinine was added to the tubes until a concentration of 1: 5,000 was attained. The blood was then added and the results compared with the control series. In all cases haemolysis commenced in the quinine series in a concentration 0·05–0·06 p.c. higher than in the control series.

In vivo experiments were carried out as follows. The normal fragility was determined in two successive days on blood obtained by cardiac puncture of a rabbit. Quinine (50 mg./kg.) was administered by mouth for the next three days and 2½ hours after each administration blood was withdrawn and the fragility determined. The animal was then allowed to recover for 1–3 days, and on two successive days blood was again withdrawn and tested. In some cases quinine was again administered and the animal allowed to recover again.

The results are given in Table 2. They show that *in vivo* quinine increases cellular fragility.

TABLE 2.*

Rabbit No.	Control period.		Quinine treatment.			Rest period.		2nd quinine treatment.		Control period.
Day	1	2	3	4	5	7	8	9	10	14
II	42	42	42	47	44		42		44	Died
III	42	42	44	42	46	43	43	44	46	44
IV	43	45	44	46	46	42	42	46	50	42
V	43		45	46	47	44	42	46	48	44
VII	43	42	Died							
IX	40	41	Died							
X	44	44	44	44	48	42				
XI	43	44	45	44	47					
XII	44	46	46	46	48	44				

Deaths were due to accident at the time of cardiac puncture.

* The figures represent the concentration in gm. per 10 litres of H_2O of sodium chloride at which haemolysis commenced.

Quinine and Hyperthyroidism.

The statement has been made (Goodman and Gilman, 1941) that persons suffering from hyperthyroidism are resistant to quinine. As far as can be determined there is no experimental evidence favourable to this hypothesis.

Two series of 12 rats each were taken. One served as a control, to the other 10 mg./kg. thyroxin was administered hypodermically for 10 days. The LD_{50} of quinine was then administered to each series. There was no significant difference in the mortality in each series.

The Influence of Raised Blood Calcium on Quinine Toxicity.

The injection of parathyroid hormone or the feeding of calcium acetate and vitamin D for 7 days did not alter the LD_{50} of quinine for rats. However in the case of the parathyroid series the convulsions were less pronounced than those in the control.

Toxicity Experiments.

(i) *To see if lowering of calcium ions increases the toxicity of quinine.* In a series of 24 rabbits it was established that the LD_{50} of 2·5 p.c. sodium oxalate was 2·5 ml./kg. (intravenously). In another series (30 rabbits) it was established that 40 mg./kg. of quinine intravenously killed only 2 rabbits.

To 15 rabbits 2·3 ml./kg. of oxalate was administered. They were divided into 3 groups and quinine was administered 1 hour, 2 hours and 3 hours later respectively. One rabbit in the 1 hour group died and two in the 2 hour group.

It is not felt that these results are significant and the experiment was not repeated because sodium oxalate precipitates quinine and it was thought that there may be a reaction in the blood stream which would interfere with the exhibition of the antagonism.

(ii) *To see if the previous administration of quinine increased the toxic effect of oxalates, presumably by lowering the calcium ion content of the cell.* A series of 20 rabbits was given 2·0 ml./kg. 2·5 p.c. sodium oxalate, all survived. 40 mg./kg. of quinine (see above) were administered intravenously on 4 successive days. Five hours after the last dose of quinine 2 ml./kg. 2·5 p.c. sodium oxalate was given; 15 rabbits died.

DISCUSSION.

It is felt that the foregoing experiments show that there is an inter-relationship between calcium and quinine. The experiments on the isolated intestine and tibialis anticus in which the results obtained with quinine parallel those given by oxalate, citrate, etc., suggest that it is one of antagonism. As the possibility of a chemical reaction between calcium and quinine is remote, the antagonism is probably one of a physiological nature. At present no mechanism is postulated, but it is interesting to recall that quinine is a well known inhibitor of enzyme action (mainly lipases) and that calcium ions may be essential for certain of these actions (they are certainly activators for adenyl pyrophosphatase (Bailey, 1943).

There are further examples of the similarities in the action of quinine and the lack of calcium ions. Quinine delays the clotting of blood. The hyperglycaemia produced in rabbits by an injection of 0.1 mg. adrenaline or 0.1 gm. calcium is arrested by 0.02 gm. quinine (Arakawa, 1933). It is well known that quinine arrests ciliary movement and it is a striking feature that of all the anions only citrates and tartrates, both of which reduce calcium ions, produce a similar inhibition, as is shown in Table xiii of Gray (1928).

The action of quinine on striated muscle in so far as it may be explained by an antagonism towards calcium must be discussed at length. The action of quinine on a neuro-muscular system is extremely complex (Harvey, 1939). Very little is known of the effect of excess or lack of calcium on muscular contraction. However, Bailey (1943) has shown that calcium ions are necessary for the activation of adenyl pyrophosphatase and presumably the absence of calcium would considerably influence muscular activity. Eccles (1942) has shown that transmission at the end-plate is dependent upon the presence of calcium. Quinine has a potentiating effect on a single maximal induction shock (this is the effect described in this paper) of such a kind that the mechanical twitch and concomitant action potential are both increased in strength and duration. Harvey states that the action cannot be due to the entry of additional fibres nor to repetitive response. It is due to increased duration of the action potential in each fibre, and as single fibre potential waves are not simultaneous but succeed one another in a manner involving a large overlap, an increase in the time each fibre is "possessed" by the action potential will clearly increase this overlap and therewith the amplitude as well as the duration of the summed action potential. Our results have shown that citrate (i.e. lack of Ca) and quinine have an identical action in this respect but the interconnection btween this and the necessity of calcium for the breakdown of adenyl pyrophosphate is obscure. Quinine, however, does depress the tetanic response to a series of shocks and this can reasonably be supposed to be due to an antagonism between quinine and calcium.

The fact that quinine exerts a mild curare-like depression at the motor endplate may be related to Eccles's observation that calcium ions are necessary for transmission. Our experiments with citrate bear this out because the first effect of the injection of citrate (apart from a brief contracture) is the abolition of response on stimulation of the nerve.

Whilst it has been relatively easy to show the similarity between the action of quinine and a deficiency of calcium ions it has been more difficult to show an an-

tagonism between quinine and excess calcium. This has been accomplished in the case of smooth muscle and the sartorius preparation, but not with the perfused frog's heart, the gastrocnemius nor certain toxicity experiments. The explanation of this is simple if we assume that quinine acts intracellularly because, as is well known, calcium does not readily penetrate the cell membrane and an excess without the cell does not necessarily mean an increased concentration within. Therefore, an increased amount of quinine would not be necessary in the presence of excess extra-cellular calcium. Support for this conception is provided by the fact that where calcium is known to penetrate the cell as in smooth muscle (Steinbach, 1940), excess calcium does antagonize quinine.

As has been stated in the introduction, the primary object of this work has been to obtain an hypothesis for the mode of action of quinine on malarial plasmodia. It is believed that the foregoing experiments do provide such an hypothesis. Namely, that calcium, an element essential for growth and irritability, in the presence of quinine is rendered unavailable to the growing plasmodia. This suggestion will now be examined in relationship to some of the known facts of malariology.

It is generally held that *in vitro* quinine is ineffective unless large concentrations are present. This means that blood treated with quinine for 24 hours is still effective on inoculation. It does not mean that *in vitro* quinine does not check the reproduction and growth of plasmodia because it is questionable whether these changes take place *in vitro*. On the other hand, several hours after the injection of quinine certain changes are observed in the plasmodia (Nauck, 1936). It is also definite that certain stages of the reproductive cycle occur in the red blood cell. It is difficult to imagine these stages taking place in the entire absence of such an essential element as calcium. Yet the amount of calcium present in the erythrocyte is so small that it cannot be determined. Therefore, the calcium of the red cell must be a limiting factor in the growth and reproduction of the protoza in the body. So that any substance capable of penetrating the red cell and interfering with the trace of calcium present must break the reproductive cycle. This it is believed is the function of quinine.

This view is supported by two observations. Firstly, that quinine is ineffective as a prophylactic if administered during the incubation period. James (1931) has found the explanation of this difficult. A solution has been suggested along the lines of an exoerythrocytic stage, similar to that which is supposed to occur in birds (Hewitt, 1941). However, the fact remains that in the above case and probably in prophylaxis in general and between relapses quinine is ineffective unless the plasmodia are dividing within the red cell. Secondly, both quinine and atebrine do not increase the rate of disappearance of parasites from the peripheral blood, but cause only a reduction in the size of the schizonts so that fewer microzoites are formed (Beckman, 1941). This interference with reproduction within the red cell we would attribute to a reduction of the already scarce calcium.

Two further generalizations lend support to the calcium hypothesis. The first is that malaria is a disease of the young, at which age the calcium of the cell is supposed to be higher than normal; and the second that malaria occurs most frequently during those seasons when the blood calcium is at its highest, i.e., spring, summer and autumn. Indeed, James has shown that sporozoites injected into humans just before winter remain dormant until the following spring (Schüffner *et al* 1930).

The inter-relationship of calcium and adrenaline in the body is too well known to necessitate the citation of examples here. Is it more than a coincidence then that we find both these substances associated with quinine in malariology? Adrenaline plays a dual role. Firstly, an injection of adrenaline will make patent a latent malaria, especially so in bird malaria; this is assumed to be due to a mechanical

action on the capillaries, but definite proof is lacking. Secondly, adrenaline has been used in the treatment of malaria, either alone (Ascoli's Method), or in conjunction with quinine. The association of calcium and malaria is not so definite, but there are several references in the literature to the use of calcium chloride as a provocative test for latent malaria whereby the multiplication of parasites can be started afresh. Interesting also is the report that quininism can be countered by the administration of calcium (Gueliadow, 1931).

Pavlova (1934) has reported that the subcutaneous injection of quinine raises the serum calcium by 14 p.c. in rabbits. The explanation of this may be that quinine reduces the concentration of calcium ions in serum and the body responds by a mobilization of calcium, and hence a rise in total serum calcium.

Sinton (1931) has suggested that the curative effect of quinine is aided by the administration of alkali and citrate, both of which would tend to lower blood calcium and according to our hypothesis be additive to the effect of quinine.

It is suggested that these facts all support the hypothesis already stated.

SUMMARY.

The addition of excess calcium ions to an isolated intestine causes a relaxation. If the intestine is previously treated with quinine or other anti-malarials the intestine now responds to calcium with a contraction. A similar "calcium reversal" is obtained if the intestine is bathed with calcium-free Tyrode or if it is treated with citrate or oxalate.

The quinine alkaloids and atebrine increase the tension developed by striated muscle. Citrates, tartrates and oxalates have a similar effect.

The usual rise in blood pressure obtained on intravenous injection of calcium is changed to a fall if the animal has been previously quininized.

Quinine increases corpuscular fragility.

Quinine increases the toxic effect of oxalates.

The hypothesis is put forward that quinine interferes with the life cycle of the plasmodia of malaria by reducing the amount of calcium available in the red blood corpuscle for growth and reproduction.

Other evidence from the literature is brought forward to support this hypothesis.

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